

18 October 2013 | \$10

Science





page 307



page 312

EDITORIAL

- 289 Science Demystified
Bruce Alberts and Marcia McNutt

NEWS OF THE WEEK

- 294 A roundup of the week's top stories

NEWS & ANALYSIS

- 297 Stunning Skull Gives a Fresh Portrait of Early Humans
>> Research Article p. 326; Slideshow
- 298 Molecule and Market Studies Capture Nobel Laurels
- 300 U.S. Shutdown Disrupts Long-Term Ecosystem Studies
>> Science Podcast
- 301 Sleep: The Brain's Housekeeper?
>> Report p. 373
- 302 South Korea's Charge Into Basic Research Meets Resistance
- 303 Botanists Spread the Gospel That Breadfruit Can Be Manna

NEWS FOCUS

- 304 Roving Into Martian Waters
- 307 Dr. Cool

LETTERS

- 310 China's Rapid Urbanization
X. J. Yang
- Gain-of-Function Research: Unproven Technique
A. Mahmoud
- Gain-of-Function Research: Unknown Risks
F. Rey et al.

- 311 CORRECTIONS AND CLARIFICATIONS

BOOKS ET AL.

- 312 Life at the Speed of Light
J. C. Venter, reviewed by S. Roosth
- 313 Black Code
R. J. Deibert, reviewed by L. DeNardis

POLICY FORUM

- 314 Probiotics: Finding the Right Regulatory Balance
D. E. Hoffman et al.

PERSPECTIVES

- 316 Sleep It Out
S. Herculano-Houzel
>> Report p. 373
- 317 Perovskite-Based Solar Cells
G. Hodes
>> Reports pp. 341 and 344
- 318 Path to Treat Rett Syndrome
A. K. Percy
- 320 How to Be Both Strong and Thermally Stable
S. Ramtani
>> Report p. 337
- 321 Did the Denisovans Cross Wallace's Line?
A. Cooper and C. B. Stringer
>> Science Podcast
- 323 Soft Acoustic Metamaterials
T. Brunet et al.

ON THE WEB THIS WEEK

>> Science Podcast

Listen to stories on a fungus deadly to frogs, Denisovans in Southeast Asia, the U.S. government shutdown's effect on science, and more.

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COVER

Photo of a 1.77-million-year-old complete adult skull (braincase volume: 546 cubic centimeters) of early *Homo* from the site of Dmanisi, Georgia. Together with the fossilized bones of four additional individuals discovered in close proximity, the skull indicates that populations of early *Homo* comprised a wider range of morphological variation than traditionally assumed, which implies a single evolving lineage with continuity across continents. See pages 297 and 326.

Photo: © Guram Bumbiashvili/Georgian National Museum

DEPARTMENTS

- 288 This Week in *Science*
- 290 Editors' Choice
- 292 *Science* Staff
- 381 New Products
- 382 *Science* Careers

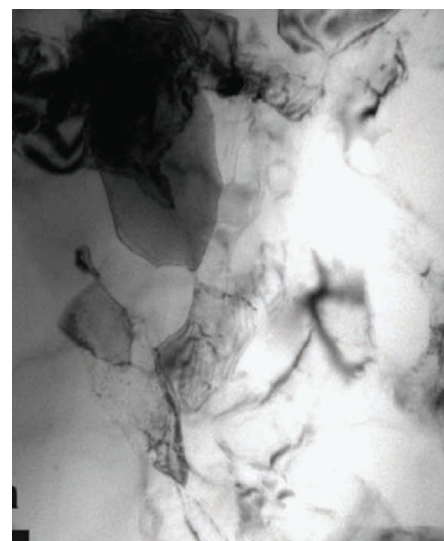
RESEARCH ARTICLES

- 325** Hyperdominance in the Amazonian Tree Flora
H. ter Steege et al.
Amazonia harbors 16,000 tree species, 1.4% of which account for half of all individual trees.
Research Article Summary; for full text:
<http://dx.doi.org/10.1126/science.1243092>
- 326** A Complete Skull from Dmanisi, Georgia, and the Evolutionary Biology of Early *Homo*
D. Lordkipanidze et al.
An early Pleistocene adult skull illuminates the evolution and morphology of the first hominins outside Africa.
>> News story p. 297

REPORTS

- 331** Stellar Spin-Orbit Misalignment in a Multiplanet System
D. Huber et al.
Kepler observations show that stellar spin-orbit misalignments are not confined to planetary systems with hot Jupiters.
- 334** Gravitational-Wave Limits from Pulsar Timing Constrain Supermassive Black Hole Evolution
R. M. Shannon et al.
Analysis of pulsar timing data sets constraints on the gravitational-wave background produced by pairs of massive black holes.
- 337** Strain-Induced Ultrahard and Ultrastable Nanolaminated Structure in Nickel
X. C. Liu et al.
Heavy shearing can produce a stronger, more thermally robust nickel microstructure.
>> Perspective p. 320
- 341** Electron-Hole Diffusion Lengths Exceeding 1 Micrometer in an Organometal Trihalide Perovskite Absorber
S. D. Stranks et al.
- 344** Long-Range Balanced Electron and Hole-Transport Lengths in Organic-Inorganic $\text{CH}_3\text{NH}_3\text{PbI}_3$
G. Xing et al.
Spectroscopy pinpoints facile carrier diffusion as a key factor in the efficiency of perovskite solar cells.
>> Perspective p. 317
- 347** Product-to-Parent Reversion of Trenbolone: Unrecognized Risks for Endocrine Disruption
S. Qu et al.
Phototransformation of growth steroid metabolites is readily reversible in aquatic environments.

- 351** Direct Spectroscopic Characterization of a Transitory Dirhodium Donor-Acceptor Carbene Complex
K. P. Kornecki et al.
A long-postulated reactive chemical intermediate has been observed and characterized.
- 355** Low Upper Limit to Methane Abundance on Mars
C. R. Webster et al.
Data from the Curiosity rover reveal an upper limit on Mars of only 1.3 parts per billion by volume for atmospheric methane.
- 357** Genomically Recoded Organisms Expand Biological Functions
M. J. Lajoie et al.
Bacteria engineered to use nonstandard amino acids show increased resistance to bacteriophage attack.
- 361** Probing the Limits of Genetic Recoding in Essential Genes
M. J. Lajoie et al.
Thirteen codons could be removed from all essential ribosomal protein-coding genes across 80 *Escherichia coli* strains.
- 364** Rapid Adaptation to Climate Facilitates Range Expansion of an Invasive Plant
R. I. Colautti and S. C. H. Barrett
Invasive populations of purple loosestrife in eastern North America have evolved increased fitness at the invasion front.
- 366** The Invasive Chytrid Fungus of Amphibians Paralyzes Lymphocyte Responses
J. S. Fites et al.
The global spread of a fungal disease of frogs may be explained by its ability to inhibit immune clearance.
>> Science Podcast
- 369** Measuring Chromatin Interaction Dynamics on the Second Time Scale at Single-Copy Genes
K. Poorey et al.
Formaldehyde cross-linking kinetics reveals rapid, site-specific transcription factor binding dynamics in vivo.
- 373** Sleep Drives Metabolite Clearance from the Adult Brain
L. Xie et al.
During sleep, metabolic waste products are removed from the extracellular spaces in the brain.
>> News story p. 301; Perspective p. 316
- 377** Reading Literary Fiction Improves Theory of Mind
D. C. Kidd and E. Castano
Experimental evidence suggests that reading good fiction helps us to understand others.



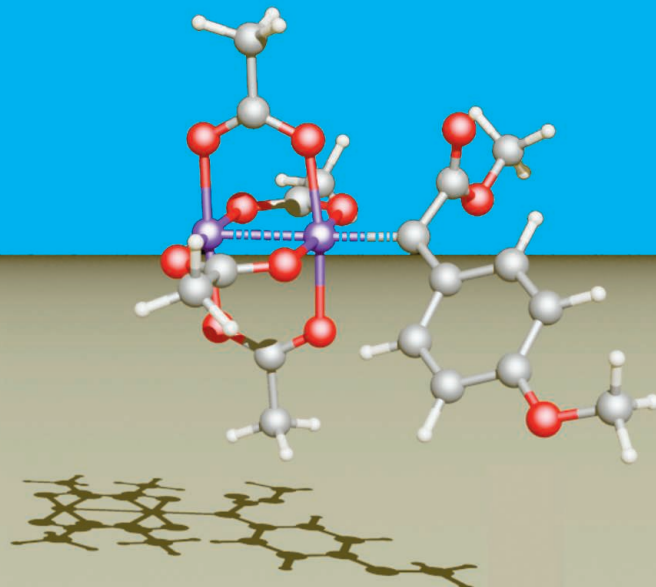
pages 320 & 337



page 366

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Catching a Carbene >>

Divalent carbon fragments, or carbenes, vary widely in their stability, depending on their substituents. Some, such as *N*-heterocyclic carbenes, are independently isolatable. Others can be isolated in coordination complexes with metals. **Kornecki *et al.*** (p. 351, published online 12 September) synthesized a carbene coordinated to a rhodium dimer representative of an elusive class of short-lived intermediates long postulated to underlie a series of cyclopropanation and C-H insertion reactions.

A Heady Find

In the past two decades, excavations at the archaeological site at Dmanisi, Georgia, have revealed hominin fossils from the earliest Pleistocene, soon after the genus *Homo* first dispersed beyond Africa. **Lordkipanidze *et al.*** (p. 326; see the cover) now describe a fossil cranium from the site. Combined with mandibular remains that had been found earlier, this find completes the first entire hominin skull from this period.

Testing Black Holes

Gravitational waves, predicted by General Relativity, are expected to be produced when very massive bodies, such as black holes, merge together. **Shannon *et al.*** (p. 334) used data from the Parkes Pulsar Timing Array project to estimate the gravitational wave background produced by pairs of supermassive black holes (those with masses between 10^6 and 10^{11} that of the Sun) in merging galaxies. The results can be used to test models of the supermassive black hole population.

Unrestricted Travel in Solar Cells

In the past 2 years, organolead halide perovskites have emerged as a promising class of light-harvesting media in experimental solar cells, but the physical basis for their efficiency has been unclear (see the Perspective by **Hodes**). Two studies now show, using a variety of time-resolved absorption and emission spectroscopic techniques, that these materials manifest relatively long diffusion paths for charge carriers energized by light absorption. **Xing *et al.*** (p. 344) independently assessed (negative) electron and (positive) hole diffusion lengths and found them well-matched to one another to the ~100-nanometer optical absorption depth.



Stranks *et al.* (p. 341) uncovered a 10-fold greater diffusion length in a chloride-doped material, which correlates with the material's particularly efficient overall performance. Both studies highlight effective carrier diffusion as a fruitful parameter for further optimization.

A Good Read

Theory of Mind is the human capacity to comprehend that other people hold beliefs and desires and that these may differ from one's own beliefs and desires. The currently predominant view is that literary fiction—often described as narratives that focus on in-depth portrayals of subjects' inner feelings and thoughts—can be linked to theory of mind processes, especially those that are involved in the understanding or simulation of the affective characteristics of the subjects. **Kidd and Castano** (p. 377, published online 3 October) provide experimental evidence that reading passages of literary fiction, in comparison to nonfiction or popular fiction, does indeed enhance the reader's performance on theory of mind tasks.

Taking Out the Trash

The purpose of sleep remains mysterious. Using state-of-the-art in vivo two-photon imaging to directly compare two arousal states in the same mouse, **Xie *et al.*** (p. 373; see the Perspective by **Herculano-Houzel**) found that metabolic waste products of neural activity were cleared out of the sleeping brain at a faster rate than during the awake state. This finding suggests a mechanistic explanation for how sleep serves a restorative function, in addition to its well-described effects on memory consolidation.

Return of the Steroid

Trace levels of organic contaminants enter aquatic ecosystems from a variety of sources, including runoff of from agricultural lands. When these compounds and their metabolites break down, it is generally assumed that they become inert and pose less ecological risk. **Qu *et al.*** (p. 347, published online 26 September) tracked the sunlight-mediated transformation of metabolites of trenbolone acetate (TBA)—a common growth-promoting steroid given to beef cattle—across a number of conditions in the laboratory and in the field. When the degradation products were exposed to dark conditions following photodegradation, they surprisingly reverted back to TBA metabolites, including analog steroidal compounds similar to TBA with unknown biological effects.

Changing the Code

Easily and efficiently expanding the genetic code could provide tools to genome engineers with broad applications in medicine, energy, agriculture, and environmental safety. **Lajoie *et al.*** (p. 357) replaced all known UAG stop codons with synonymous UAA stop codons in *Escherichia coli* MG1655, as well as release factor 1 (RF1; terminates translation at UAG), thereby eliminating natural UAG translation function without impairing fitness. This made it possible to reassign UAG as a dedicated codon to genetically encode nonstandard amino acids while avoiding deleterious incorporation at native UAG positions. The engineered *E. coli* incorporated nonstandard amino acids into its proteins and showed enhanced resistance to bacteriophage T7. In a second paper, **Lajoie *et al.*** (p. 361) demonstrated the recoding of 13 codons in 42 highly expressed essential genes in *E. coli*. Codon usage was malleable, but synonymous codons occasionally were nonequivalent in unpredictable ways.

Additional summaries

Seeing the Trees in the Forest

Despite botanical exploration over two centuries, knowledge of the species composition and quantitative distribution of the trees of the Amazonian forest has remained decidedly patchy.

Ter Steege *et al.* (p. 325) report the results from a network of 1170 tree plots arrayed across the Amazon Basin and Guiana Shield, in



which the species of all trees with stem diameter >10 centimeters were identified. The tree flora comprised a total of about 16,000 species. However, just 227 very common Amazonian species accounted for half of the trees in the Amazon—the world's most diverse forest.

Misaligned Planets

Stars with multiple coplanar planets have not been seen to show misalignments between the equatorial plane of the star and the orbital plane of the planets—a diagnostic of the dynamical history of planetary systems. **Huber *et al.*** (p. 331) analyzed the Kepler 56 planetary system, which contains a giant-sized and an intermediate-sized planet. The planets have orbits that are close to coplanar, but the planetary orbits are misaligned with the stellar equator. A third companion in a wide orbit, which could be another star or a planet, could explain the misaligned configuration.

Taking the Strain

When heavily deformed through compressive or torsional loading, crystalline metals will generate an increasing density of defects or dislocations that effectively strengthens the metal against further deformation. However, at some stage, the fine-grained structure that forms saturates. **Liu *et al.*** (p. 337; see the Perspective by **Ramtani**), show that combining the application of a very-high-rate shear deformation with high strain gradients to the surface layer of a pure sample of nickel can overcome this saturation. Instead of a three-dimensional fine-grained structure, a top layer with a two-dimensional layered structure occupied the first 80 micrometers. In addition to being stronger, this layered nickel structure was also more thermally stable.

No Methane to Be Found

On Earth, atmospheric methane is mostly produced biologically. Atmospheric methane has also been detected on Mars, but these reports have been controversial. Based on data from the Sample Analysis at Mars instrument suite on the Curiosity rover, which arrived at the surface of Mars in August 2012, **Webster *et al.*** (p. 355, published online 19 September) report no methane, with an upper limit of only 1.3 parts per billion by volume, about 6 times lower than previous measurements.

Invade and Adapt

The mechanisms by which plant and animal species spread into new habitats have become an increasing focus of ecological research, particularly in the context of climate change and species invasions. **Colautti and Barrett** (p. 364) examined the ecological consequence of local adaptation evolving rapidly along a 1000-kilometer climatic gradient in purple loosestrife

(*Lythrum salicaria*), one of the most notorious invasive plant species in North America. These invasive populations have evolved to become locally adapted within 50 to 100 years with important ecological consequences—increasing reproductive output by more than an order of magnitude.

Breaking Frog Defenses

The first line of immune defense against most fungal infections consists of innate immune effector cells, including macrophages and neutrophils. However, **Fites *et al.*** (p. 366) have found that the fungus currently decimating the world's amphibians, *Batrachochytrium dendrobatidis*, is readily engulfed by these cells, but that this does not effectively control the infection. The fungus releases cell-wall components that induce lymphocyte apoptosis and inhibit the proliferation of other nonlymphoid cell types, disarming lymphocyte-mediated responses to infection.

Capturing Binding Location and Speed

Transcription factor-binding sites in chromatin can be mapped by the chromatin immunoprecipitation (ChIP) assay, which analyzes formaldehyde-fixed chromatin fragments obtained from cells. However, the standard ChIP assay does not provide information about how stable the inter-actions are. Other approaches, including live-cell imaging, can reveal aspects of the dynamic behavior of transcription factors but are limited either in location precision or time resolution. **Poorey *et al.*** (p. 369, published online 3 October) developed a model to explain how the ChIP signal relates to formaldehyde cross-linking time, and they developed a method to measure chromatin site-specific binding dynamics with high temporal resolution.



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Marcia McNutt is Editor-in-Chief of *Science*.

Science Demystified

WE ARE PLEASED TO ANNOUNCE A NEW RESOURCE FROM *SCIENCE* FOR EXPOSING PRE-COLLEGE and university students to the nature of science as a fascinating and powerful way of knowing about the world. The need to do so is by no means new.* “Being well informed about science is not the same thing as understanding science...What is needed is methods for importing some knowledge of the tactics and strategy of science to those who are not scientists.”† Our new initiative, *Science in the Classroom (SitC)*, is designed to help demystify how scientists build a basis for understanding the world. It is freely available (<http://scienceintheclassroom.org>) as a resource to enable more effective teaching and understanding of the sciences.

The products of science and technology have the potential to improve health and livelihoods, counteract environmental threats, and provide adequate drinking water and food to the nearly 10 billion people expected in the increasingly crowded world of 2050. For a nation to take optimum advantage of what science has to offer, it is imperative that its citizens understand how scientists make judgments and gain confidence in the scientific approach for evaluating the safety of foods and of medical treatments, warning of dangers to the global environment, or arguing for immediate actions that address potential crises. Experience demonstrates that myths can very easily spread when a population lacks a solid understanding of how scientific knowledge is generated. At both the individual and community levels, this lack of appreciation can lead to important decisions being made that are based not on science, but on what might be called “magical thinking.”

SitC has been designed to help every educated person attain an understanding of the nature of scientific knowledge by reading at least one scientific paper before he or she completes school. On the open-access *SitC* Web site, an initially small set of published science research papers has been posted. Each publication includes a set of tools to facilitate student exploration and understanding. A “Learning Lens” tool highlights and clarifies special vocabulary, for example, and figures are deconstructed into parts that are annotated with explanations, as well as with questions for students to ponder. Many of the authors’ references are annotated so that students can follow how scientists build on previous scientific findings.

To help connect each scientific publication to the students’ lives, free links to relevant News and Policy articles in *Science* are provided. Designed by outstanding teachers, Teacher’s Guides present several alternative ways to use *SitC* as a teaching resource. At their suggestion, an abbreviated version of each paper is presented in parallel with the full university version. *SitC* also connects users to the *Science* Education Portal, where a great deal of other *Science* content relating to science education is freely available. The entire *SitC* project, developed with funding from the United States National Science Foundation, owes a great deal to the feedback provided by many dozens of teachers throughout the project’s evolution.

Science is launching *SitC* with six publications that cover biology, chemistry, and physics; future publications will expand both the topics and disciplines covered, giving teachers a large range of subject matter from which to choose. As emphasized in the *SitC* Teacher’s Guides, each paper is designed to be used to illustrate general points about the way that science is done and the nature of scientific communication. Thus, the exact topic covered in class is less important to us than the fact that students will be exposed to an authentic science paper and learn how the authors use evidence to derive important new understandings. And for some, this encounter could ignite a passion to pursue science as a career.

— Bruce Alberts and Marcia McNutt

*J. A. Moore, *Science As a Way of Knowing (SAAWOK)*, Society for Integrative and Comparative Biology (www.sicb.org/dl/saawok.php3). †J. B. Conant, *Science and Common Sense* (Yale Univ. Press, New Haven, CT, 1951).



EVOLUTION

Push-Me, Pull-You

Natural selection is expected to shape phenotypes around an adaptive optimum. In cases where the trend is expected to be directional—bill lengthening in birds, for example—the trait under selection should move toward this optimum. In reality, however, traits often vacillate around it. One cause for this lack of movement is opposing selection. From a 25-year study of green-rumped parrotlets in Venezuela, Tarwater and Beissinger describe evidence for opposing selection as a causal factor. Females that reproduced early in the year produced more offspring; however, both the females and their offspring had lower survival in the following year, indicating a trade-off between selection for fecundity and that for viability. Further, these patterns were oppositely influenced by environmental conditions. Rainfall strongly selected for early breeding, whereas breeding density favored later breeding. Climate change could induce a declining positive-feedback loop wherein small numbers of offspring would be produced due to low rainfall, and this would lead to increased selection for viability and continued low recruitment. —SNV

Proc. Natl. Acad. Sci. U.S.A. **110**, 10.1073/pnas.1303821110 (2013).



APPLIED PHYSICS

Ridge-Riding Plasmons

Nanophotonics aims to integrate the speed of optics with the nanometer size scale of the electronics industry. The several-orders-of-magnitude difference in size scale of the respective components present a technological challenge to that integration. Surface plasmons, the sub-wavelength collective light-induced electronic excitations that propagate at the surface of metals, can bridge that size gap and so are an area that is being actively pursued. However, there tends to be a trade-off between how far the plasmons can propagate and the extent of their confinement. Using a silicon-on-insulator platform with a silver overlayer, Mu *et al.* present a simulation study showing that forming a ridge

in the silicon can help relax the restrictions of that trade-off. By varying the geometry of the ridge, they show that the confinement of the plasmons can be enhanced without compromising their propagation length. The compatibility of their structure with conventional electronics processing techniques also lends itself favorably to the development of integrated optoelectronic circuits and devices. —ISO

Appl. Phys. Lett. **103**, 131107 (2013).

CANCER

TAMing Gliomas

Tumor-associated macrophages (TAMs) facilitate tumor growth by stimulating cancer cell proliferation, by helping cancer cells evade the immune system, and by promoting tumor angiogenesis.

In human cancer patients, high levels of TAMs often correlate with poor prognosis. These cells are thus an exciting target for new cancer therapies, most of them designed to destroy TAMs. In a twist on this concept, Pyonteck *et al.* describe a promising therapy that works not by destroying TAMs but by “reeducating” the cells so that they lose their tumor-promoting functions and instead acquire tumor-suppressive functions.

In mouse models of glioma, a brain tumor that is resistant to most therapies, the authors found that administration of a brain-penetrant inhibitor of colony-stimulating factor 1 receptor (CSF-1R), a signaling protein that regulates macrophage differentiation, resulted in tumor regression. In the setting of CSF-1R inhibition, TAM survival was promoted by cytokines secreted by the glioma cells. The inhibitor produced a characteristic gene expression signature in the mouse TAMs; in TAMs found in one subtype of human glioma, the same pattern correlated with increased patient survival. —PAK

Nat. Med. **19**, 10.1038/nm.3337 (2013).

BIOCHEMISTRY

Stepwise Energetics

One fact that has been drilled into legions of introductory biochemistry students is that the hydrolysis of adenosine triphosphate (ATP) produces energy that is yoked to other reactions. What is less generally inculcated is that some of this energy can be extracted when ATP binds to proteins, in the form of conformational change, and that this can drive reactions too. In a study of nitrogenase, Duval *et al.* illustrate these two processes in a series of elegant kinetic measurements. They find that the interaction of the ATP-bound Fe protein and the MoFe protein triggers sequential electron transfer from the P cluster to the M cluster (both in the MoFe protein) and then from the Fe-S cluster in the Fe protein to the P cluster. Only after these transfers take place is the ATP hydrolyzed, and only after P_i is released can the ADP-bound Fe protein let go of the MoFe protein. This cycle repeats until the eight electrons needed to effect dinitrogen reduction (and hydrogen evolution) have been delivered. The distinction between binding and hydrolysis will remind biochemists of a certain age of the actomyosin ATPase. —GJC

Proc. Natl. Acad. Sci. U.S.A. **110**, 10.1073/pnas.1311218110 (2013).

EDUCATION

Going Backwards

For decades, many U.S. communities have bused students to schools beyond their local neighbor-

hood, a result of Supreme Court decisions aimed at reducing racial segregation. Recent legal challenges have led to the elimination of busing in many areas, with students' schools again determined by the neighborhoods in which they live. In Charlotte-Mecklenburg, North Carolina, busing was eliminated, but school-assignment boundaries were also redrawn, changing which neighborhoods sent students to these schools. Billings *et al.* took advantage of this natural experiment to study educational and social outcomes. They tracked over 40,000 students, roughly half of whom changed schools as a result of remapping. The black-white student achievement gap widened, and both black and white students scored lower on exams after assignment to new schools with larger proportions of minority students. White students were less likely to graduate high school or attend a 4-year college when assigned to schools with more minority students. Finally, crime rates among minority males increased. Allocating more resources to high-minority schools may have offset some of the negative academic outcomes at earlier ages, suggesting policy remedies to counter negative peer effects in school. — BW

Q. J. Econ. **129**, 10.1093/qje/qjt026 (2013).



PHYSICS

Intrinsic Conductor

The unexpected finding of a conducting interface between two insulators, LaAlO_3 (LAO) and SrTiO_3 (STO), can be explained by extrinsic mechanisms such as the formation of oxygen vacancies; whether this interface is intrinsically conducting is still an open question. Warusawithana *et al.* designed an experiment to minimize the influence of extrinsic factors and glean the influence of the stoichiometry of the LAO layer on the conductivity of the interface. Their mosaic sample was formed by cutting several STO substrates into four pieces each and mounting the pieces from the different substrates next to each other; an LAO layer was then grown on top using molecular beam epitaxy where the ratio of the La and Al fluxes was finely varied to accomplish a gradient of LAO stoichiometries across the mosaic. The authors found that the conducting samples were Al-rich. In these conditions, the excess Al substituted for the missing La, and no vacancies were formed; the natural

discontinuity in polarization at the interface was relieved by the formation of a two-dimensional conducting layer. In contrast, in La-rich samples the discontinuity was neutralized by the migration of cations from the interface, enabled by vacancies created because of the off-stoichiometric composition. Similar mechanisms may be at work in other interfacial systems. — JS

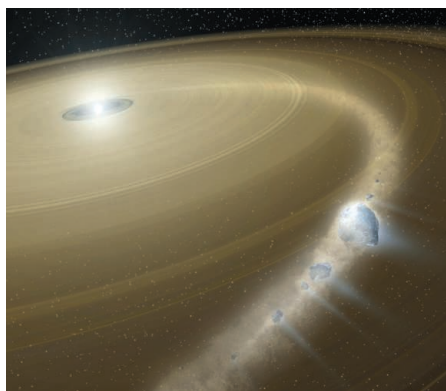
Nat. Commun. **4**, 2351 (2013).

ASTRONOMY

An Extrasolar Perspective

White dwarfs represent the end stage in the evolution of Sun-like stars. There is evidence that the atmospheres of some of them contain material from planetesimals shredded after their orbits passed too close to the dying star. Jura *et al.* examined the iron-to-aluminum abundance ratio in the atmospheres of seven white dwarfs with well-measured abundances and found that this ratio varies by more than a factor of 100 across the sample. The authors attribute this variation to igneous differentiation, which concentrates iron in cores and aluminum in crusts, and to impacts and collisions between planetesimals, which vary the iron-to-aluminum ratio.

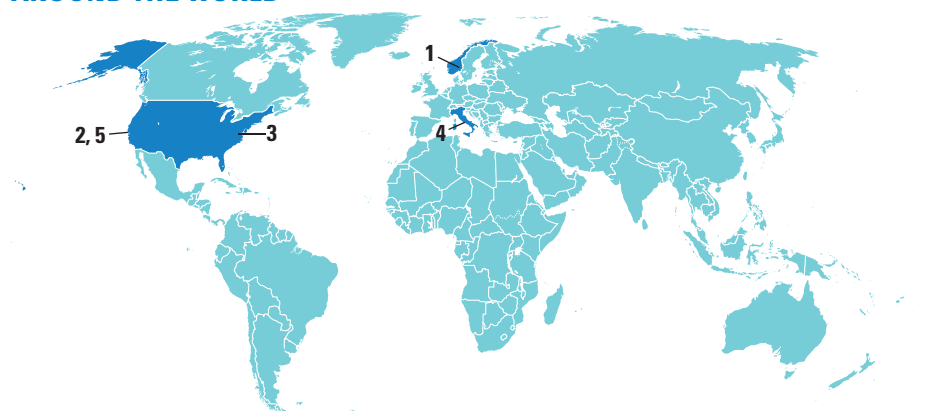
The heat source for igneous differentiation must have come from the radioactive decay of ^{26}Al —a radioisotope that was an important short-term heat source in the early solar system and which is produced within massive stars and injected into the interstellar medium



through stellar explosions or stellar winds. The inferred abundance of ^{26}Al in the extrasolar environments where the shredded planetesimals formed is similar to that inferred for the early solar system. Thus, the conventional view that our solar system was unusual in its initial ^{26}Al abundance may not be justified. — MJC

Astrophys. J. **775**, L41 (2013).

AROUND THE WORLD



Oslo 1

Chemical Weapons Watchdog Group Wins Peace Prize

For its “extensive efforts to eliminate chemical weapons,” the Nobel Committee has chosen to honor the Organisation for the Prohibition of Chemical Weapons (OPCW) with the 2013 Nobel Peace Prize.

“We are a small organisation which for over 16 years, and away from the glare of international publicity, has shouldered an



Honored. Ahmet Üzümcü, OPCW's director-general, comments on receiving the Nobel Peace Prize.

onerous but noble task—to act as the guardian of the global ban on chemical weapons that took effect in 1997,” said OPCW Director-General Ahmet Üzümcü in a prepared statement.

Such international bodies are not famed for their speed and effectiveness, but OPCW's track record is impressive. Some 189 countries, representing 98% of the global population, have joined OPCW since the Chemical Weapons Convention came into force in 1997 and 82% of the world's

declared stockpile of chemical agent (some 58,172 tonnes) has been destroyed. OPCW has carried out 5286 inspections at 228 chemical weapon–related and 1905 industrial sites in 86 countries. OPCW's recent mission in Syria, following the August chemical weapon attacks in Damascus, has brought it much greater public attention. <http://scim.ag/chemweapnobel>

Mountain View, California 2

NASA Backs Away From Meeting Ban

The Chinese scientists banned from an upcoming meeting at NASA's Ames Research Center will get another chance to register (*Science*, 11 October, p. 177). But that doesn't guarantee them a seat at next month's conference.

The turnaround, announced last week by NASA Administrator Charles Bolden, comes after an angry letter from Representative Frank Wolf (R–VA), who chairs the panel that funds the space agency. NASA Ames officials said they were simply following language Wolf inserted into a 2011 spending bill with the goal of protecting NASA against industrial and military spying by China. But the real reason was a blanket ban that Bolden had imposed in March on visits by scientists from eight countries, including China.

Wolf asked Bolden to “correct the record,” and 2 days later Bolden extended his olive branch without acknowledging any mistake. Instead, Bolden blamed “[m]id-level managers at Ames ... performing the due diligence ... following a period of significant concern and scrutiny from Congress.” The scientists will still need to pass a security clearance, however, a process that generally takes several weeks.

Richmond 3

Climate Heats Up Virginia Politics

A new poll suggests that the issue of climate change may help businessman Terry McAuliffe in his campaign for governor of Virginia next month against Ken Cuccinelli, the outspoken state attorney general.

McAuliffe, a Democrat, leads Cuccinelli, a Republican, by nine points, according to the media outlet POLITICO, which polled 1150 likely voters. Cuccinelli, who questions the scientific consensus that global warming poses a significant threat, has criticized McAuliffe's support for new federal



Hot topic. Climatologist Michael Mann has campaigned for Terry McAuliffe, Ken Cuccinelli's rival for Virginia governor.

rules on carbon emissions from coal plants. But the poll showed 45% in support of the regulations, versus 33% opposed.

Meanwhile, climate scientist Michael Mann released a video last week saying that the attorney general was “doing the bidding of the fossil fuel interests” when he issued a 2010 subpoena of Mann's e-mails and other scientific documents. The subpoena, centered on grant applications Mann had filed during a previous stint at the University of Virginia, was eventually tossed out by a state judge. Now at Pennsylvania State University, University Park, Mann campaigned in support of McAuliffe earlier this year.

NOTED

>The Boston-based Institute for Justice & Democracy in Haiti filed a lawsuit last week against the United Nations for **inadvertently unleashing the 2010 cholera outbreak** that has killed more than 8000 Haitians. Genomic studies and an independent U.N. report left little doubt that Nepalese peace-keeping forces brought the disease to the island nation.

CREDITS: (TOP LEFT TO RIGHT) GREG RICO; STEVE HELBER/AP PHOTO; (BOTTOM) GREG DEJONG/AP PHOTO

Downloaded from www.sciencemag.org on October 17, 2013



Remnant of a Comet

A black, diamond-spackled pebble just a few centimeters across is the remainder of a comet that struck Earth almost 29 million years ago—making it the first direct evidence of a comet exploding in our atmosphere, scientists say. The stone, named “Hypatia” after an Alexandrine mathematician and philosopher, was found in 1996 among bits of yellow sand glass (also known as the Libyan Desert Glass) scattered across tens of kilometers in southwestern Egypt, near the border with Libya. The glass itself has been dated to 28.5 million years and has long been thought to be the result of a meteorite impact or an airburst caused by a comet breaking up in Earth’s atmosphere. Scientists performed a range of tests on the tiny pebble, examining its mineralogy, bulk chemistry, carbon isotope, and noble gas content—which support not only an extra-terrestrial but also a cometary origin, they report in an upcoming issue of *Earth and Planetary Science Letters*.

THEY SAID IT

“Oh, what news?”

—Theoretical physicist Peter Higgs, to a former neighbor who hailed him from a car to congratulate him on “the news”—that he would share the Nobel Prize in physics for his work on the theory of the Higgs boson.

fuel, aiming to fuse the hydrogen atoms into helium atoms, releasing energy. The goal is ignition, a self-sustaining fusion burn that produces more energy than the laser put in.

The BBC story reported that during one recent experiment, “the amount of energy released through the fusion reaction exceeded the amount of energy being absorbed by the fuel - the first time this had been achieved at any fusion facility in the world.” But although a 29 September NIF memo to collaborating labs describes a promising fusion shot that produced 75% more neutrons—a product of fusion—than any previous shot, it is not the breakthrough everyone is hoping for. Ignition, scientists say, is still a long way off. <http://scim.ag/noNIFbreak>

NEWSMAKERS

Three Q's



Choi

To rejuvenate a sagging economy, South Korean President Park Geun-hye is looking to her nation’s scientific corps to tickle its creativity bone and produce more innovations. Samsung Elec-

tronics heeded the call, announcing in August that it will establish a 500 billion won (\$466 million) foundation to hand out grants for basic research. The government, meanwhile, hopes to get creative juices flowing through its Institute for Basic Science (IBS), which provides generous support to handpicked top talents (see p. 302). Leading the charge is science minister **Choi Mun-kee**, an expert on information and communications technology. Choi, 62, spoke with *Science* on 4 October in his office in Seoul.

Q: How influential will Samsung’s foundation be in coaxing scientists to innovate rather than imitate?

C.M.-K.: We expect that Samsung could show us a new model that connects basic, >>

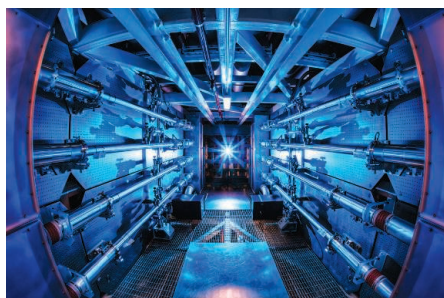
identify “has already given the green light to human experiments.”

<http://scim.ag/Staminablocked>

Livermore, California 5

The NIF Breakthrough That Wasn't

One side effect of the U.S. federal shutdown is that press officers at government labs aren’t around to offer reality checks to news stories. Last week, media outlets jumped on a report by BBC News that the National Ignition Facility (NIF) at Lawrence Livermore National Laboratory in California had passed a “nuclear fusion milestone.” NIF uses the world’s highest energy laser system to crush tiny pellets containing hydrogen



No ignition. NIF “breakthrough” was overhyped.

Rome 4

Italy Blocks Use of Stem Cell Therapy

Italy’s Health Minister Beatrice Lorenzin announced on 10 October that Stamina, a foundation in Turin that developed a controversial and unproven stem cell therapy, will not be allowed to test it on humans—at least not in Italy. “We have put an end to it,” Lorenzin says. “I would have liked to give patients better news, but the method has shown to be not eligible for a clinical trial.”

The treatment is based on bone marrow stem cells that Stamina’s President Davide Vannoni claims can grow neurons and cure neurodegenerative diseases. In May, the government provided €3 million for a clinical trial. However, last month, an expert panel unanimously rejected the method, claiming that it lacks scientific basis (*Science*, 20 September 2013, p. 1324). A Health Ministry decree explains that the rejection was based on an “inadequate description of the method” and “the lack of quality controls on cells.”

The Justice Ministry has yet to rule on whether Stamina can continue to treat patients in Italy. In the meantime, Vannoni says, an African country that he declined to

>>NEWSMAKERS

applied, and development research. This is a starting point. We hope others will follow.

Q: Why did the government establish IBS rather than strengthen existing centers of excellence in the universities, for example?
C.M.-K.: Korea has suffered greatly from brain drain. We have established IBS to create a good environment for brain collection.

Q: In recent months, scientific cooperation between North and South Korea has virtually ceased. With tensions on the Korean Peninsula easing, do you see an opportunity for science diplomacy?

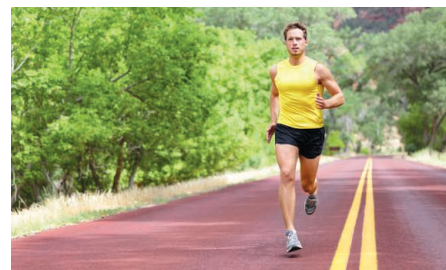
C.M.-K.: We are very much interested in cooperating. For example, I'd like to see a

national park in the DMZ [demilitarized zone]. Cooperation in science will be no problem, if we have enough trust.

FINDINGS

Protein Pathway Links Exercise To Brain Health

Exercise has well-known mental benefits, from counteracting depression and aging to fighting Alzheimer's and Parkinson's diseases. Scientists have struggled to account for this—but research published last week in *Cell Metabolism* reveals an important molecular link: a protein called FNDC5, produced in muscle cells during exercise and released into the bloodstream in a form called irisin. FNDC5 is also present in the



Pumped up. A group of molecules link exercise to mental benefits.

brain and is thought to help neurons develop.

The researchers found that in mice, exercise increases FNDC5 in the hippocampus, a brain region responsible for learning and memory. Ramping up FNDC5 production in mouse brain cells developing in a dish boosted levels of a crucial protein called brain-derived neurotrophic factor (BDNF), involved in maintaining healthy neurons and creating new ones. Most surprisingly, the researchers say, injecting mice with a virus that causes their livers to secrete more irisin also increased BDNF in the hippocampus, suggesting that irisin, or some unidentified protein that it regulates, could be crossing the blood-brain barrier to work its effects.

<http://scim.ag/WorkoutBrain>

Random Sample

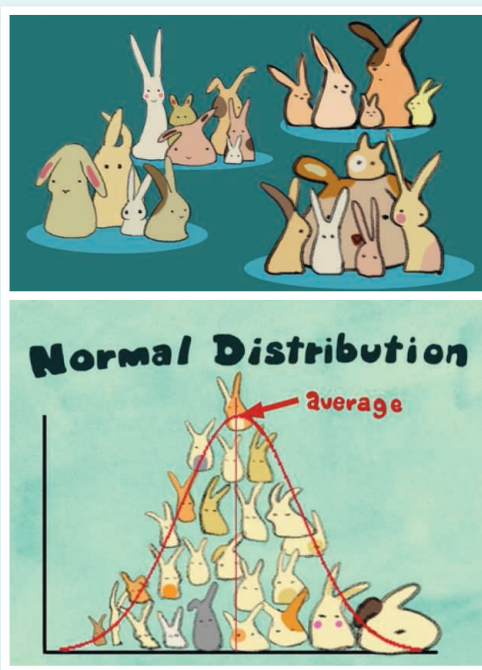
Animals Get the Spotlight In CreatureCast

Invertebrates, such as jellyfish, worms, or mollusks, don't have the same charismatic appeal as your average lion or tiger, so those who want to share their interest in the creatures often resort to the "ick factor," says Brown University evolutionary biologist Casey Dunn. "But that serves to create a distance between the organism and the audience, which is contrary to what you're trying to do."

Dunn is trying something different with CreatureCast, a series of 3-minute animated videos largely starring invertebrates. In 2009, Dunn and some friends received a National Science Foundation grant to study mollusk phylogeny and part of it was allocated to communicating their findings. CreatureCast (<http://creaturecast.org>) videos are produced with limited resources, often by a single writer/editor/producer who is also a student of Dunn's. (Students in his Invertebrate Biology class at Brown can produce a video as a final project.) Instead of expensive photos, the videomakers rely on hand-drawn animations, in which "imperfections are endearing instead of being a liability," Dunn notes.

Although invertebrates dominate the video subjects, one recent effort tackled a more abstract statistical topic (explained with cartoon bunnies and dragons): an explanation of the central limit theorem (pictured), which states that under certain conditions, and with a sufficiently large sample size, the averages of samples will have an approximately normal distribution.

The videos are now poised to hit the big time, thanks to a partnership that *The New York Times* initiated this summer. Each week, the paper considers available videos and may select one to appear on the online science page. "It's been really great," Dunn says. "I have no background in storytelling, and now I'm starting to bounce ideas off of editors."



Potential Bioweapon Kept Secret

Sometimes, transparency in science must take a back seat to security concerns. That's what the editors of *The Journal of Infectious Diseases* decided in the case of two papers reporting the discovery of a new type of botulinum toxin. Fearing that that information could be exploited by terrorists seeking bioweapons, the editors and the authors of the papers, published online last week, decided that it was prudent not to publish the sequence of the bacterial gene that produces the protein, named Botulinum Toxin H.

Antitoxins exist for the seven previously discovered botulinum toxins (with suffixes A to G), but the new toxin did not respond to any of these. In an editorial accompanying the papers, the journal said that the gene sequence would be made public in the future, after an antitoxin is developed.

Science LIVE

Join us on Thursday, 24 October, at 3 p.m. EDT for a live chat about new approaches for treating **spinal cord injuries**.
<http://scim.ag/science-live>



PALEOANTHROPOLOGY

Stunning Skull Gives a Fresh Portrait of Early Humans

It was the ultimate birthday gift. On his 42nd birthday, 5 August 2005, paleoanthropologist David Lordkipanidze was presented with the most complete early *Homo* skull ever found, freshly uncovered at his site of Dmanisi, Georgia. As paleontologists gently brushed dirt off its face, they saw strange, primitive features, unexpected in even an early member of our genus: a protruding, apelike upper jaw, and a tiny braincase. One scientist joked that “you should put it back in the ground,” Lordkipanidze recalls.

Instead, Lordkipanidze and an international team spent 8 years studying the fossil, which they describe on page 326. Dating back to about 1.8 million years ago, the spectacular skull includes delicate parts of the face, rare in other finds, making it “the world’s first completely preserved adult hominid skull” of such antiquity, they write. Combined with skulls found earlier at Dmanisi, it also suggests that ancient individuals from the same time and place were very different from each other but still members of one species—an idea that has implications for the perplexing patchwork of *Homo* fossils found in Africa.

The skull “is undoubtedly one of the most important ever discovered,” says paleoanthropologist Ian Tattersall of the American Museum of Natural History in New York City. “An iconic fossil,” proclaims Tim White, a paleoanthropologist at the University of California, Berkeley. “It will stand out for a long time.”

Over the past 20 years, Lordkipanidze and his colleagues have unearthed skulls and

skeletal bones from at least five individuals from Dmanisi, a site that preserves the oldest human fossils found outside of Africa. These early humans used crude stone tools, probably for butchering animals, and lived near a river, a busy watering hole, says geoarchaeologist Reid Ferring of the University of North Texas, Denton. Ferring used argon isotopes to date the site to 1.77 million to 1.85 million years ago, showing that *Homo* had expanded into Asia not long after the genus appeared in Africa.

At that time about 18% of the site’s animal bones belonged to carnivores, including fierce saber-toothed cats and an extinct giant cheetah. Confrontations with these beasts would have been common—and dangerous. All five individuals were found in underground dens where carnivores had probably dragged their carcasses. Ferring thinks the skeletons were all deposited “within a couple centuries, at most,” after which the dens collapsed.

This carnivores’ cache has produced new fossils season after season. The massive lower jaw of the new skull was found back in 2000, and Lordkipanidze and others had expected to unearth a huge cranium to go with it. Instead they found the cranium of an older man with arthritis in his jaw, worn front teeth probably used as a gripping tool—and a small brain of only 546 cubic centimeters. That’s within range of an earlier human ancestor, *Australopithecus*, whose brains averaged 450 cm³, compared with 1350

Putting their heads together. Researchers think that all five of these skulls from Dmanisi belong in one species. Skull 5 is on the right.

cm³ for modern humans, notes co-author Christoph Zollikofer, a neurobiologist at the University of Zurich in Switzerland. This confirms that our ancestors didn’t need big brains to get out of Africa.

When the researchers attached the lower jaw to the new cranium, designated Skull 5, the lower face bones jutted out more like an *Australopithecus*. “This is, in essence, a very primitive face,” says co-author Yoel Rak of Tel Aviv University in Israel.

Yet it’s clearly *Homo*, many researchers agree. The skull’s vertically oriented upper face and the shape of the braincase



distinguish it from *Australopithecus*. Skeletal bones found with the skulls, including Skull 5, show that although these humans were short in stature, they had modern body proportions and could walk long distances (*Science*, 21 September 2007, p. 1664).

But what species of *Homo* is it? Some fossils previously discovered at Dmanisi seemed to have links to *H. erectus*. But when the big lower jaw was found in 2000, some researchers suggested it belonged to a new species they called *Homo georgicus*.

With the discovery of the new, fifth skull,

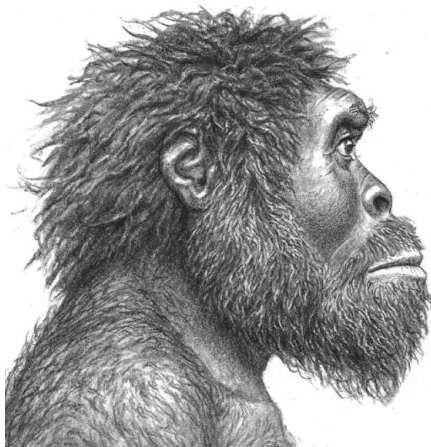
Online

sciencemag.org

See slideshow for images of the new skull (http://scim.ag/slide_6156).

the researchers had to confront head-on the variation among all five. Age and sex probably account for much of it: The skulls are thought to have belonged to an elderly toothless male, two mature males, a young female, and an adolescent of unknown sex. This broad sample from one place and a short span of time is what makes Dmanisi an “exceptional site,” White says. By analyzing the skull shapes with 3D computer-based methods, the researchers found that the range of variation in the group at Dmanisi was no greater than within living humans or chimps. The team concluded that all five skulls belong to a single, variable species.

Putting all five skulls into a single species still left the problem of what to call it. The team squabbled at first. Looking at particular traits, they found that the upper jaw of Skull 5 most closely resembles the oldest fossil proposed as *Homo*—a 2.3-million-year-old jaw from Ethiopia tentatively assigned to *H. habilis*. But Skull 5 also shares key features with *H. erectus*, such as thick brow ridges. In the end, the team settled on the cumbersome moniker of *Homo erectus ergaster georgicus*, which recog-



Lowbrow. This artist's reconstruction shows the new skull's small brain and protruding jaw.

nizes the skull as an earlier Georgian form of *H. erectus*. But they all prefer to call their finds “early *Homo*.”

The skull shape analysis and classical trait analysis, done by Zollikofer and his Zurich colleague Marcia Ponce de León, also showed that the skulls were as variable as African fossils traditionally classified in three different species—*H. erectus*, *H. habilis*, and *H. rudolfensis*. If the Dmanisi fossils had been found in separate places in Africa, they could have been called separate species, Ponce de

León says. Lumping them all into *H. erectus* suggests that the early *Homo* fossils in Africa may also belong to that same, single lineage.

That controversial idea is setting off a small “bomb” in the field, says co-author Philip Rightmire of Harvard University. Tattersall thinks Dmanisi could include more than one species, and that Skull 5 represents a new species. Paleoanthropologist Ron Clarke of the University of the Witwatersrand in Johannesburg, South Africa, counters that Skull 5 “looks to me like *Homo habilis*.” And while paleontologist Fred Spoor of the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, thinks it “sensible” to call Skull 5 *H. erectus*, he balks at the notion that fossils in Africa all belong in *H. erectus*, too, arguing that the team's analyses can't delineate diagnostic differences in skull shape.

Whatever Skull 5's specific identity, Spoor agrees that it is a “fantastic, terrific specimen.” Says White: “No matter what you call it, this skull and the others from Dmanisi are some of the best evidence we have about how, where, when, and why humans evolved.”

—ANN GIBBONS

PRIZES

Molecule and Market Studies Capture Nobel Laurels

A Prize for Molecular Modeling

You've seen the models—molecules represented as balls connected by sticks, often pared down to a few lines on paper. Useful as they are to chemists, they leave out something essential: motion, the intricate dance of electrons and atomic nuclei breaking and fusing bonds as they undergo reactions. That dance is the heart of chemistry. Last week's Nobel Prize in chemistry was awarded to three U.S.-based scientists for developing computer models that reveal how proteins and other compounds perform it.

All three of this year's chemistry laureates are naturalized U.S. citizens. Martin Karplus of Harvard University and the University of Strasbourg in France was born in 1930 in Vienna and moved to the United States just before the outbreak of World War II. Michael Levitt of Stanford University's School of Medicine in Palo Alto, California, was born in Pretoria in 1947,

and today is a British, U.S., and Israeli citizen. And Arieh Warshel of the University of Southern California in Los Angeles was born in 1940 in Kibbutz Sde-Nahum, Israel, and still holds Israeli citizenship.

More than 40 years ago, the three pioneered new tools for fusing two disparate world views among chemists working to simulate molecules. One of them, grounded in classical Newtonian physics, treated molecules as collections of atomic balls connected

by springlike bonds. Because this approach was mathematically tractable for large numbers of atoms, it enabled researchers to simulate proteins and other large molecules. In 1969, Levitt and Warshel, then both at the Weizmann Institute of Science in Rehovot, Israel, designed a ball-and-spring computer model that could track how proteins and other large biomolecules oscillate and twist. But it couldn't calculate the changes in energy involved when chemicals react and

form new molecules.

Meanwhile, at Harvard, Karplus was deeply enmeshed in the second approach to simulation, called quantum chemistry. It was far better at simulating the motion of the electrons and atomic nuclei involved in reactions. But it was so computationally demanding that it was useful only in solving the behavior of small molecules.

Trying to bring the quantum and classical



worldviews together “was a fairly natural progression,” Levitt told reporters last week. In 1970, Warshel visited Karplus’s lab and brought his classical program with him. The two soon constructed a program that melded their approaches, treating mobile, covalent bond-forming electrons (pi electrons) with a quantum chemical approach and less mobile ones (sigma electrons) as well as atomic nuclei classically. They used their hybrid approach to calculate the behavior of simple organic molecules.

In 1976, Warshel and Levitt followed up with a more general approach and showed that it worked for simulating the behavior

of the protein lysozyme. They used quantum chemistry to handle lysozyme’s reactive core but a classical approach to deal with more distant parts of the molecule. The strategy—akin to devoting extra pixels to the focal point of an image to improve its resolution—remains at the heart of computational chemistry. “The prize recognizes developments that started over 40 years ago that still reverberate today through much of chemistry and biology,” says Klaus Schulten, a molecular modeling expert at the University of Illinois, Urbana-Champaign.

Today, those reverberations include hybrid “multiscale models” capable of sim-

ulating more than 4 million atoms, which are revealing the complexities of processes as diverse as the translation of genes into proteins and the photosynthetic conversion of sunlight into chemical fuel. Thousands of researchers are hard at work in the field. Still, Schulten says, the Nobel Committee chose well: “If you had to single somebody out, I think there’s a good case to be made that these three were the right choice.”

Even so, Warshel says he was wary when he reached for the ringing phone at 2 a.m. on 9 October: “I checked to see if they talked in a Swedish accent just to be sure.”

—ROBERT F. SERVICE

Theories of Stock Pricing Win Big

Being able to predict how stock prices will rise or fall, everybody knows, is worth a fortune. Understanding how stock prices can be predicted in the first place can also net you a pretty rich dividend, as Eugene Fama, Lars Peter Hansen, and Robert Shiller can now attest. The three have together won the 2013 Sveriges Riksbank Prize in Economic Sciences in Memory of Alfred Nobel (often informally called the Nobel Prize in economics) “for their empirical analysis of asset prices.” Fama and Hansen are professors at the University of Chicago; Shiller is a professor at Yale University.

“These are three men whose work I have been studying, learning from, and grappling with my whole professional life,” says John Campbell, an economist at Harvard University who wrote his dissertation under Shiller years ago. “Some people are surprised at this way of honoring them jointly, but to me it makes great sense.”

In the 1960s, Fama and his colleagues showed that it was impossible to predict stock prices in the short term. He noted that they quickly respond to new information—such as tougher environmental regulations affecting the oil industry—leaving little scope for predictions of future changes. Fama’s findings led to the emergence of index funds, which spread risk over many stocks, allowing investors to avoid spending time and effort to try determining how individual stocks may fare over days and weeks.

In the 1980s, Shiller showed that although Fama was right, there was more to the story.

When Shiller studied stock prices relative to the dividends earned by the stocks over the years, he found a pattern. In theory, stocks should rise and fall year by year, depending on the dividends they paid, but what actually happened, Shiller found, was that when stocks were priced higher than their dividends could justify, they fell in price in the following years in a way that made stock prices predictable over a 3- to 7-year horizon.

stand the complex behavior of markets.

Forecasting the price trajectory of stocks, real estate, and other assets remains a shaky science. The collapse of the U.S. real estate market 6 years ago and the global financial crisis that followed show that economists cannot always predict catastrophic trends and respond accordingly. Still, the work of Fama, Hansen, and Shiller has paved the way for many follow-up studies aimed at understand-

ing why the market doesn’t always do a good job of assigning the right price to assets, leading to bubbles and crashes.

Shiller says he hopes that the recognition he and his two co-laureates have won will bring more attention to these questions and to the field of finance more broadly. “I believe that finance is a theory that, while it has many controversial elements, also has a body of knowledge that is useful to society and will improve

human welfare,” he said, speaking to reporters briefly by phone at the Nobel Committee’s press conference announcing the prize. Asked about the recent financial crisis, he said it reflected “mistakes and imperfections in our financial system” that policymakers and institutions were now correcting.

Shiller was the only one of the new laureates to speak at the press conference. Fama appears to have been busy preparing for a class he was scheduled to teach later in the morning. When *Science* called his home a couple of hours after the announcement, Fama’s wife, Sallyann, answered to say that he was at his department and unavailable. “He said nothing was going to interfere with his class,” she said. —YUDHIJIT BHATTACHARJEE



In 1982, Hansen developed a statistical method for testing the theory of how the savings and investment decisions of individuals affect stock prices. His work led to the understanding that economists were wrong to assume that people behave as rational actors who base their decisions solely on expected future returns. In bad times, investors become much more cautious than usual; in good times, irrational exuberance can lead them to take risks that cold calculations would bar. Their pessimism and optimism make stock prices less predictable than earlier models had assumed. Hansen’s work, along with the work of Shiller and others, laid the foundations of behavioral finance, a field that is helping economists to under-

POLICY

U.S. Shutdown Disrupts Long-Term Ecosystem Studies

Nature doesn't stand still. That reality has amplified the impact of this month's U.S. government shutdown for scientists conducting long-term ecosystem studies.

Scientists accept having peers pull the plug on their research as part of the merit review process. They also know that some interruptions are an inevitable part of doing fieldwork. But they bristle at the fact that a political brawl—even if Congress reaches a deal this week—has jeopardized their ability to sustain decades-old time-series data sets used to tease out subtle shifts in the natural world.

In Antarctica, for instance, last week a team of scientists learned that their 23-year-old study of how sea ice fluctuations affect polar biota could miss an entire field season. The first wave of researchers had arrived at Palmer Station on the Antarctic Peninsula to monitor thousands of penguins that later this month will begin a 5-month-long breeding season. The peninsula's penguin communities are changing fast: The native Adélie population crashed in the late 1990s and is down to about 2000 breeding pairs, while Gentoo penguins, a non-native species whose range is expanding because of a warming climate, have become dominant.

But instead of preparing for twice-weekly visits to the dozens of colonies on off-shore islands, the scientists were told they would be sent home this week unless the shutdown had ended. "In the past, bad weather might have meant we lost days or weeks of data," says Hugh Ducklow, a biological oceanographer at Columbia University who directs the project. "But we've never had an entire year's interruption."

The Antarctic research is taking place at one of 26 Long Term Ecological Research (LTER) sites funded by the National Science Foundation (NSF). And while the Palmer LTER has money in the bank, the project is on hold because NSF can't pay the contractor that handles logistics for the three U.S. Antarctic stations.

The effects may continue even after funding is restored. Ducklow was planning to arrive at Palmer in late December to lead a 5-week research cruise along the peninsula collecting oceanographic data to correlate sea ice fluctuations with the birds' breeding cycles. However, the cruise is on the U.S. research icebreaker *Laurence M. Gould*, the same ship that ferries scientists and supplies between Chile and Palmer. It's anybody's guess whether Ducklow's team will retain its place on the *Gould's* schedule after the shutdown ends.

Another LTER scientist feeling the pinch is ecologist Scott Collins of the University of New Mexico, Albuquerque. His project,

climate variability affects the soil's ability to sequester carbon over many years. This year, Collins may not be able to collect data to help solve that puzzle. "If we wait too long," he says, "the plants will have started dying and the data will be goofy."

The shutdown is also interrupting LTERs in Antarctica's Dry Valleys and in Florida's Everglades National Park; both sites are off limits. The Everglades LTER also works closely with National Park Service scientists now on furlough, and the service provides boat and helicopter access to many sampling sites. "Their workload will be more than doubled when they get back," predicts Evelyn Gaiser, an ecologist at Florida International University in Miami who leads the project, about her federal partners.

Many LTER projects use automated sensors to collect data. But those instruments sometimes need to be tended. Last month, for example, feral dogs killed a Barbary sheep near one of Collins's instruments on the Sevilleta. The savage attack uprooted several components and left a mess for researchers. "You just never know what could go wrong," Collins says.

LTER scientists don't expect their projects to continue forever, of course. For ecologist John Moore, who directs the Natural Resource Ecology Laboratory at Colorado State University in Fort Collins, the end came 3 years ago, when NSF didn't renew funding for his Shortgrass Steppe LTER project in northern Colorado, which began in 1982.

Since then, Moore and his team have used close-out money to "adjust our sampling schedules to fit our reduced resources." His experience may have made him a bit more sanguine than his LTER colleagues about surviving a data disruption.

"I can guarantee you that everybody has had a gap for some reason—a storm, a vacation, a temporary loss of funding, or whatever," he says. "And if people are honest with themselves, they will admit that it's not the end of the world." But he adds, "it's never desirable to have gaps in your data."

—JEFFREY MERVIS



A scientific drought? A rainfall manipulation experiment at the shuttered Sevilleta LTER site in New Mexico.

based at the nearby Sevilleta National Wildlife Refuge, sits on land managed by the National Park Service, which has shut down. "Closed means closed," refuge managers told him when he asked for an exemption.

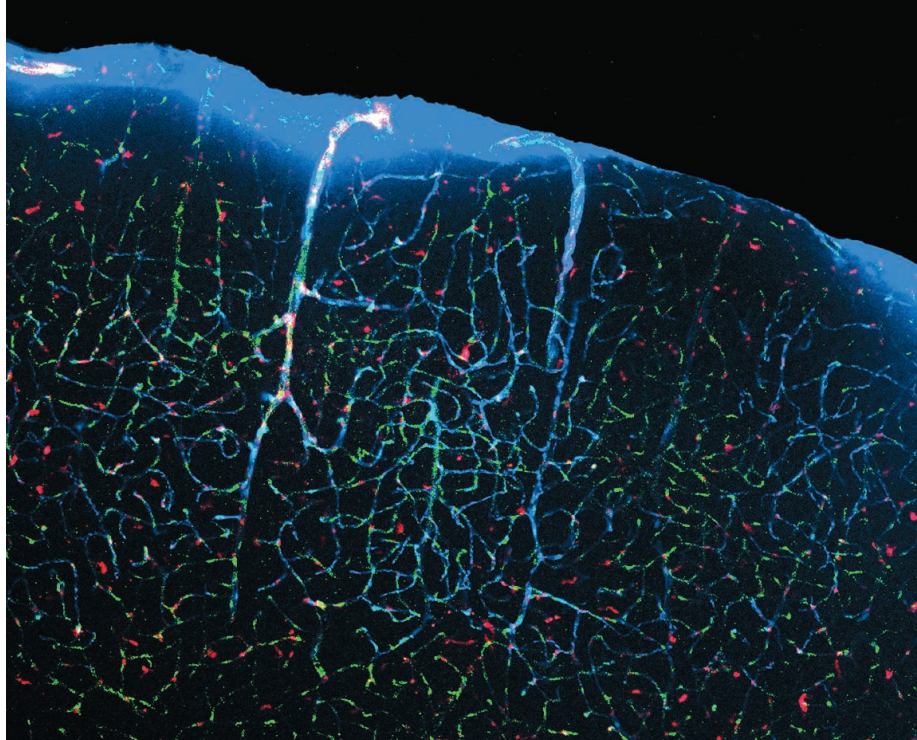
As with Ducklow, the timing of the shutdown couldn't have been worse for Collins, who leads a project begun in 1988 on how flora and fauna in this arid region fluctuate in response to rainfall. This summer's monsoon season was 50% heavier than normal, triggering explosive plant growth and other changes in the ecosystem. Normally, his team would be busy tallying the biomass increase and censusing small mammals, data that would be compared with similar information taken in previous years.

But counting prairie dogs isn't the real point. The project's larger goal is to trace how

Online

sciencemag.org

Podcast interview with author Jeffrey Mervis (http://scim.ag/pod_6156).



NEUROSCIENCE

Sleep: The Brain's Housekeeper?

Every night since humans first evolved, we have made what might be considered a baffling, dangerous mistake. Despite the once-prevalent threat of being eaten by predators, and the loss of valuable time for gathering food, accumulating wealth, or having sex, we go to sleep. Scientists have long speculated and argued about why we devote roughly a third of our lives to sleep, but with little concrete data to support any particular theory. Now, new evidence has refreshed a long-held hypothesis: During sleep, the brain cleans itself.

Most physiologists agree that sleep has come to serve many different purposes, ranging from memory consolidation to the regulation of metabolism and the immune system. While the “core” purposes of biological functions such as breathing and eating are easy to understand, however, scientists have never agreed on any such original purpose for sleeping. The new study, by Maike Nedergaard and colleagues at the University of Rochester in New York, provides what Charles Czeisler, a sleep researcher at Harvard Medical School in Boston, calls the “first direct experimental evidence at the molecular level” for what could be sleep’s basic purpose: It clears the brain of toxic metabolic byproducts.

The new work, reported on page 373, “fits with a long-standing view that sleep is for recovery—that something is paid back or cleaned out,” says David Dinges, a sleep researcher at the University of Pennsylvania.

It builds on Nedergaard’s recent discovery, described last summer in *Science Translational Medicine*, of a network of microscopic, fluid-filled channels that clears toxins from the brain, much as the lymphatic system clears out metabolic waste products from the rest of the body. Instead of carrying lymph, this system transports waste-laden cerebrospinal fluid (CSF). Before the discovery of this “glymphatic system,” as Nedergaard has dubbed it, the brain’s only known method for disposing of cellular trash was to break down and recycle it within individual cells, she says.

In the original work, Nedergaard’s group showed that glia, the brain’s non-neuronal cells, control the flow of CSF through channels in their cell membranes. “If we delete the channels in glial cells, the flow almost stops,” Nedergaard says. Because the transport of fluid across cell membranes requires a lot of energy, Nedergaard and her team had a hunch that the brain would not be able to both clean the brain and process sensory information at the same time. So they decided to test whether the activity of the glymphatic system changed during sleep. Lulu Xie, the new study’s first author, spent the next 2 years training mice to relax and fall asleep on a two-photon microscope, which can image the movement of dye through living tissue.

Once Xie was sure the mice were asleep, based on their EEG brain activity, she injected a green dye into their CSF through a catheterlike device in their necks. After

Brainwashing. When mice sleep, fluid-filled channels (pale blue) between neurons expand and flush out waste.

half an hour, she awakened them by touching their tails and injected a red dye that the two-photon microscope could easily distinguish from the green. By tracking the movements of red and green dye throughout the brain, the team found that large amounts of CSF flowed into the brain during sleep, but not during the awake state, Nedergaard says.

A comparison of the volume of space between nerve cells while the mice were awake and asleep revealed that the glial channels carrying CSF expanded by 60% when the mice were asleep. The team also injected labeled β amyloid proteins into the brains of sleeping mice and awake mice and found that during sleep, CSF cleared away this “dirt” outside of the cells twice as quickly—“like a dishwasher,” Nedergaard says. Such proteins can aggregate as pathogenic plaques inside cells and are associated with Alzheimer’s disease, she says.

Many neurological diseases—from Alzheimer’s disease to stroke and dementia—are associated with sleep disturbances, Nedergaard notes. The study suggests that lack of sleep could have a causal role, by allowing the byproducts to build up and cause brain damage. “This could open a lot of debate for shift workers, who work during the nighttime,” Nedergaard predicts. “You probably develop damage if you don’t get your sleep.”

One unknown, however, is whether the need to remove waste products actively regulates sleep—whether, for example, the buildup of metabolic byproducts makes us sleepy. Researchers also wonder how the fluid-filled channels change shape during sleep, and whether clearing waste actually improves the function of neurons.

No one role of sleep necessarily rules them all, says sleep scientist Derk-Jan Dijk of the University of Surrey in the United Kingdom. “Sleep probably has many functions,” he says, just as the weekend is variously for shopping, socializing, and cleaning the house.

But now that Nedergaard and her colleagues have identified this nightly brainwashing in mice, Czeisler says, scientists can investigate whether it occurs in all species, and to what extent. “One could imagine that different species have evolved different additional functions of sleep to suit their different habitats, ... but this will help resolve if there is some shared function of sleep across species,” he concludes.

—EMILY UNDERWOOD

South Korea's Charge Into Basic Research Meets Resistance

DAEJEON, SOUTH KOREA—As a leader of South Korea's premier dark matter experiment, Kim Sun Kee enjoyed the hunt for an elusive quarry. So 2 years ago, when the nascent Institute for Basic Science (IBS) was looking for a director to oversee the design and construction of a heavy-ion accelerator, the physicist hesitated to apply. But the idea of bringing South Korea's biggest-ever basic research facility into being was irresistible. When Kim's team fires up the RAON Rare Isotope Accelerator around 2020 in this science city south of Seoul, the \$1 billion machine will probe how heavy elements are created in supernovas, join the race to discover stable superheavy nuclei—and symbolize Korea's new push into basic research.

South Korea spent \$45 billion on R&D in 2011, ranking it sixth in the world. Only about \$8 billion of that public and private spending went to basic research. “Until now, Korea heavily invested in short-term, result-focused applied research,” says science minister Choi Mun-kee. IBS, a government-run entity that was inaugurated last year and is slated to receive \$3.3 billion over its first 6 years, is meant to shift that focus. But where some basic researchers see a boon, others see a boondoggle. Critics have blasted IBS as a “monster” and a “black hole” that will suck resources from existing grant programs, particularly at the National Research Foundation of Korea (NRF). “Whenever there is a big change, you're bound to have resistance,” says IBS President Oh Se-Jung, a physicist who, in a town hall-style meeting in Seoul last month, sought to reassure the scientific community that IBS is a force for good.

Politicians also believe that IBS is vital to Korea's economy. In recent decades, the nation's growth strategy has been to adopt and build on foreign innovations. But growth has slowed, and “the follower model is no longer viable,” Choi says. South Korean President Park Geun-hye, an electrical engineer by training, has called for a “creative economy” based on homegrown innovations—“a paradigm shift to a first-mover model,” Choi says, in which prosperity “will depend on imagination and ideas.”

IBS's basic blueprint envisions 50 or so

research centers, each organized around a world-class scientist's area of expertise. So far, 19 center directors have been appointed, including five non-Koreans; IBS is hoping to lure many more from abroad over the next few years. Each director in turn recruits several group leaders: rising stars who aren't afraid of virgin territory. In Korea, “everybody is accustomed to safe projects. Scientists aren't taking risks,” Oh says. “Changing that culture can only be done in a new institution.”

The sole megacenter at IBS is Kim's 90-strong accelerator team. The other centers are labs that will be housed at four planned IBS complexes around the country, includ-

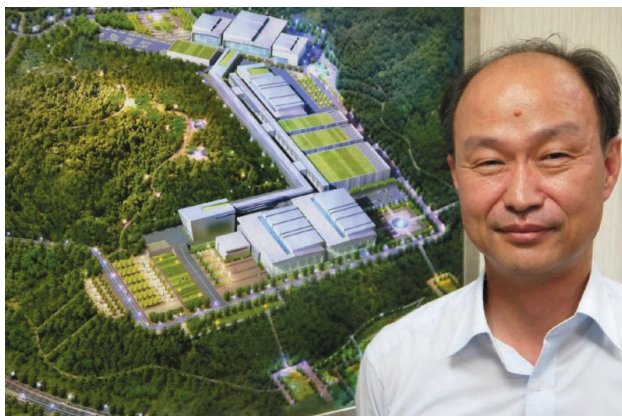
heavily on young scientists, pinching mid-career scientists. “Competition [for grants] has become fiercer,” Choi says. As success rates fell, critics lashed out at IBS—unfairly, Oh insists: “I've made it clear we will not touch the NRF budget.”

Oh's charm offensive has won some converts. One vocal critic, plant geneticist Lee Ilha of Seoul National University, says he “opposed IBS strongly” at first because he assumed it would drain funding from NRF. After speaking with Oh, Lee was reassured that's not the case—and has even warmed to IBS. “Korea has never tried such an approach before,” Lee says. “It's a new adventure.” Others are unswayed. “There's a lack of transparency,” says bioinorganic chemist Joan Valentine, a visiting professor at Ewha Womans University in Seoul who confronted Oh at the town hall meeting. Many critics are afraid to speak out, she says.

A lingering concern for many is that the group leaders may be too young and untested to make good use of their hefty budgets: as much as \$1.3 million a year. “That's too much money,” Lee says. Oh says that IBS will make the review process for hiring group leaders stricter, without, he hopes, favoring candidates with a more conservative bent. “It's a delicate balance,” he says.

Many scientists believe IBS's lasting legacy will derive from backing projects that South Korea might otherwise miss out on. One example is IBS's Center for Synaptic Brain Dysfunctions, which will scour mouse brains for defects in synaptic proteins and neural circuits associated with behaviors that correlate to diseases such as autism spectrum disorders in humans and then decipher mechanisms. The pricey project, requiring many mouse lines and imaging facilities, “would be impossible to do here without IBS support,” says center director Kim Eunjoon, a neuroscientist here at the Korea Advanced Institute of Science and Technology. The hope is that IBS will inspire the Korean scientific community as a whole to think out of the box while building a “creative economy.” “If we can change the research culture,” says deputy science minister Yang Sung-Kwang, “we can make a great leap forward.”

—RICHARD STONE



Heavy responsibility. Kim Sun Kee hopes to have the \$1 billion RAON Rare Isotope Accelerator, IBS's big science facility, fully operational by 2020.

Korea by the Numbers (2011 figures)

\$45 billion National R&D spending (6th in world)

4.03% R&D investment to GDP ratio (2nd in world)

44,718 Number of papers published in science citation index journals (12th in world)

ing a headquarters to be built on the site of the 1993 Daejeon Expo, and through extramural programs at Seoul National University and Sungkyunkwan University in Suwon. IBS initially announced that it would allot each center a whopping \$10 million a year for 10 years. It has since acknowledged that a one-size-fits-all approach isn't tenable and now will dole out funds—from \$3.7 million to \$12.1 million a year—according to each center's research plan.

Lavish support for a chosen few has inevitably drawn intense scrutiny. NRF's funding tripled to nearly \$1 billion a year between 2008 and 2012, but has flattened since then. Recent grant competitions have focused



Propagator-in-chief. Diane Ragone ships breadfruit saplings to 22 countries.

FOOD SECURITY

Botanists Spread the Gospel That Breadfruit Can Be Manna

KAUAI, HAWAII—With a reputation for being mealy and bland—a slur, say proponents—the breadfruit may seem an unlikely candidate to become the next epicurean phenomenon. But if Diane Ragone prevails, the prickly green South Pacific native will be appearing on more and more menus, especially in regions with shaky food security. That's because breadfruit is packed with nutrients, the trees are prolific, and most important, Ragone is on a mission to spread saplings far and wide.

A decade ago, Ragone, a horticulturalist, launched the Breadfruit Institute here at the National Tropical Botanical Garden (NTBG). Now home to 120 varieties from 34 Pacific islands, the institute has become something of an ark for the plant as rarer varieties wink out in their native habitats. But Ragone, its director, hopes to do more than simply conserve a little-known delicacy. The institute has developed techniques for propagating the trees efficiently and is shipping them by the thousands to Ghana, Kenya, Haiti, and 19 other countries. A new \$300,000 grant from the Ceres Trust, a nonprofit that supports research on organic agriculture, will help step up dissemination.

"This crop could be so important to people to just take care of their basic caloric needs," says Eve Emshwiller, an ethnobotanist at the University of Wisconsin, Madison, and past president of the Society for Economic Botany. The Breadfruit Institute

has found that the perennial trees produce more food in dry weight per hectare than corn, rice, or wheat. A tad smaller than a football, the fruit is rich in iron, potassium, and vitamin A precursors. And certain varieties ground into flour pack more protein than rice.

South Pacific islanders 3000 years ago stowed breadfruit and its wild ancestor, breadnut, for sea voyages as they colonized the region. A few varieties made it as far as the Caribbean in the 1700s, brought by French voyagers and by Captain William Bligh, of *Mutiny on the Bounty* fame.

Two hundred years later, Ragone went island-hopping across the Pacific, collecting hundreds of breadfruit varieties. She rescued some in the nick of time. For instance, revisiting Chuuk State in Micronesia in 2004, she says, "loss of breadfruit was pretty striking." There and elsewhere in the South Pacific, breadfruit varieties have taken hits from natural causes such as cyclones and more complex cultural ones, such as islanders abandoning traditional foods as they adopt modern life-

styles, Ragone says.

The Breadfruit Institute set out to reverse those trends, but "like all things in life, it wasn't as easy as you thought it should be," says former NTBG staffer Susan Murch, a chemist at the University of British Columbia, Okanagan, in Canada. Root cuttings were the only way to propagate the tree until the institute pioneered tissue culture techniques. Murch toiled to find a safe way to purge breadfruit roots of harmful fungi and microbes, as well as neutralize the plant's latex, which is normally sequestered in vacuoles or in the phloem but which tends to leak in tissue culture and kill dividing cells. Another challenge was finding the right combination of growth regulators and compounds to prompt cultured tissue to transform into sprouts. Diligence paid off: Murch has 28 varieties growing well.

Now the breadfruit enthusiasts have to get consumers to embrace it. "You can't just magically introduce a plant somewhere and expect it to solve all the food problems," says Nyree Zerega, a breadfruit expert at Northwestern University and the Chicago Botanic Garden. To boost the crop's appeal, the Breadfruit Institute has collected more than 300 recipes (breadfruit chips are a common favorite, at least among research-

ers) and experimented with producing breadfruit flour, which is gluten-free. Other parts of the tree have historically been used as construction materials and to make glue, fabric, and insect repellent.

Breadfruit's culinary revival could begin in NTBG's backyard. Called ulu in Hawaiian, breadfruit was once a staple on the islands but fell out of favor at least a century ago. With support from the Ceres Trust, the Breadfruit Institute in 2012 and 2013 distributed 4877 saplings throughout Hawaii. And this summer, it organized

the first Breadfruit Festival here, drawing crowds for cooking demonstrations and to buy trees. Children from a native Hawaiian charter school sang, thanking their ancestors for the gift of breadfruit.

—SARAH ZIELINSKI

Sarah Zielinski is a science writer in Washington, D.C.



Worthy of ululation. The breadfruit, or ulu, could be a boon for food security.

Roving Into Martian Waters

Will the Curiosity rover discover an early Mars that was warm and wet and life-friendly or cold and icy and inhospitable?

THE \$2.5 BILLION CURIOSITY ROVER IS on a journey to a mountain in search of the truth about ancient Mars. In the midst of the 154-kilometer-wide Gale crater, where the rover landed in August 2012, rises a 5.5-kilometer-high pile of sediment called Mount Sharp. Buried in its layers, Mars scientists believe, is a history of water on early Mars and a potential resolution to an emerging martian dispute.

Ever since the two Viking spacecraft sent home images in the 1970s of water-cut valleys on Mars, most Mars scientists have believed that during its first billion years or so, the planet was shrouded in a thick, warm atmosphere capable of raining often enough to carve out those valleys. It was an environment seemingly hospitable to the origin and sustenance of life.

But in the past decade, as the latest wave of Mars orbiters returned even sharper views of the surface, some planetary scientists have been advancing a far less life-friendly view. According to these cold-and-icy thinkers, early Mars never had a permanent atmosphere thick enough to drench the planet, even for brief intervals. There was water on the surface all right, but it was almost always tied up as ice. On this alternative Mars, the surface was icy, dry, and hostile for many millions of years at a time, and life would have struggled to gain a foothold there.

What will Curiosity find next year, as it

Destination Mount Sharp. This sediment pile holds a history of water on early Mars for Curiosity to read.

begins to climb the slopes of Mount Sharp? “That is the \$2.5 billion question,” says planetary spectroscopist Ralph Milliken of Brown University. It could be a paleoclimatologist’s dream: a neat stack of sediment layers recording a changing but mostly wet climate, and maybe even containing the organic remains of ancient martian life. Or it could be a pile of sediments altered by ground water pooled far below a dry, lifeless surface. Or it could be a total surprise. “We’ll learn something useful and interesting,” says planetary geologist Jeffrey Moore of NASA’s Ames Research Center in Mountain View, California. “But it may not be what some people hoped it would be.”

From wet to dry to wet

Mars as 19th and early 20th century astronomers envisioned it was a world of deserts, lakes, and canals, not unlike the American West. All that turned to dust when the Mariner spacecraft of the 1960s flew past Mars and sent back the first close-ups of its surface, which seemed to show a parched, cratered terrain that looked more like the moon. A decade later, however, the image of Mars changed again with the two Viking orbiters, which revealed networks of river valleys in the martian tropics. Mars was warm and wet again—at least in its early history about 4 billion years ago.

Planetary fluvial geologists, who study the shapes of erosion features to gauge how much water it took to carve them, concluded that early Mars must have had an atmosphere far thicker than today’s. By counting the number of craters that have

accumulated on martian surfaces, geologists can infer their ages—roughly when valley networks were carved, for example, or a delta deposited. They estimated that the heyday of erosion was in the Noachian period—the first several hundred million years of the martian geologic record—when enough rain fell to cut valley networks, erase craters smaller than 4 kilometers in diameter, and erode hundreds of meters of rock off of larger craters. But there couldn’t have been a lot of water sloshing around, or the craters would have overflowed.

“Early warm and wet Mars was never humid by terrestrial [Earth] standards,” says planetary fluvial geologist Rossman Irwin of the Smithsonian Institution’s National Air and Space Museum in Washington, D.C. But “it could have been semiarid. Mars in its heyday was like Utah or Nevada during the last ice age,” when Great Salt Lake was even greater and lakes abounded.

Even in the warm and wet scenario, Mars went downhill, climatically speaking, after the few hundred million years of the Noachian. Erosion rates fell, although one or more periods of hydrological “reactivation” interrupted the long drying. In those clearly wet times, flowing water formed crater lakes, built river deltas in some lakes, and washed debris off crater rims to form half-cone-shaped alluvial fans. In fact, Curiosity landed on the toe of a kilometers-high fan nestled against the rim of Gale crater, which formed around the end of the Noachian about 3.7 billion years ago.

Several hundred million years later, “Mars dies,” as Irwin’s Air and Space colleague Robert Craddock puts it. Researchers agree that for the 3 billion years since then, Mars

has remained icy and hyperarid (*Science*, 11 April 2003, p. 234).

The picture of an early Mars of at least intermittent rain was reinforced 10 years ago when the Opportunity rover landed on the floor of what was taken to be an ancient shallow, salty sea. Its twin rover, Spirit, eventually discovered hot-spring deposits like Yellowstone's.

Convincing as the evidence was to geologists, however, planetary climate modelers weren't at all sure. Their models of early Mars's climate could not produce conditions warm enough for rain or flowing water. Half again as far as Earth from a young sun that was 25% dimmer than it is now, early Mars was a perpetual ice ball, at least in the models. Even a thick carbon dioxide atmosphere could not break the planet out of its deep freeze.

That may be changing. Climate modelers had struggled to show how liquid water could have existed on early Earth, given the faintness of the newborn sun, but they have recently succeeded in producing a temperate early Earth. The key for some modelers: enriching the model planet's atmosphere with hydrogen molecules, which—at least when they collide with lots of other molecules—can efficiently absorb radiation and act as a powerful greenhouse gas.

Planetary climate scientists James Kasting, Ramses Mario Ramirez, and Michael Zugger of Pennsylvania State University, University Park, have followed that lead for early Mars. Their model tucks the planet up snugly in a thick carbon dioxide atmosphere that contains 10% hydrogen, the sort of atmosphere they believe likely enshrouded the planet back then. As the group reported at last December's meeting of the American Geophysical Union, a hydrogen-rich atmosphere would have kept the martian surface at the time of Gale crater's formation above freezing on average year-round.

Icy Mars

Planetary geologist James Head of Brown University doesn't see the need for a hydrogen blanket because he doesn't think Mars was ever permanently warm and even occasionally rainy. At last March's Lunar and Planetary Science Conference, he cited a dozen reasons from his own work and that of others for arguing that Mars was always icy and that the water world envisioned a

decade ago should go the way of the 19th century martian canals.

The eroded craters and water-cut valleys are real enough, Head says, but he thinks they could have formed during rare episodes of ice melting. That's the way the water cycle works around the year in the Dry Valleys of Antarctica, where Head does fieldwork. It's below freezing on average there, but enough ice melts in the daytime summer warmth to cut gullies in the bare earth nearby.

In Head's scheme—which he calls Noachian icy highlands—Mars had ice near its water-cut features. He and colleagues have deduced from lingering glacial features, like sediment ridges deposited in channels beneath glacial ice, that an ice sheet did indeed circle

similar climate modeling that puts snow and ice right where it was needed to supply meltwater to cut valley networks.

To melt that snow and ice, Head calls on volcanoes or possibly large impacts. Giant impacts, of which there were lots on early Mars, scatter hot ejecta globally. That vaporizes ground ice, producing a hot, steamy atmosphere that could unleash heavy rains. Such sudden warmings had seemed too short-lived, but atmospheric scientist Teresa Segura of Space Systems/Loral LLC in Palo Alto, California, and her colleagues may have found a way around that. In the July 2012 issue of *Icarus*, they showed in a model how, under the right conditions, a giant impact could drive a frigid martian climate



A wet Mars for sure. While planetary scientists debate a warm and wet versus a cold and icy early Mars, they all agree that for at least a few geologically brief intervals, Mars was awash, as in this artist's concept.

the south pole in the late Noachian and chill the southern hemisphere.

Planetary climate modelers François Forget and Robin Wordsworth of Pierre and Marie Curie University in Paris, with Head and others, showed how the ice might have formed. They ran an early-Mars climate model with a moderately thick carbon dioxide atmosphere. It could not warm the planet above freezing, but like Earth's atmosphere and unlike today's wisp of a martian atmosphere, it cooled with increasing altitude. That meant that snow could fall at the high elevations of the southern hemisphere, compacting into ice. And in the 28 August issue of *Geophysical Research Letters*, Kathleen Scanlon of Brown, Head, and colleagues report on

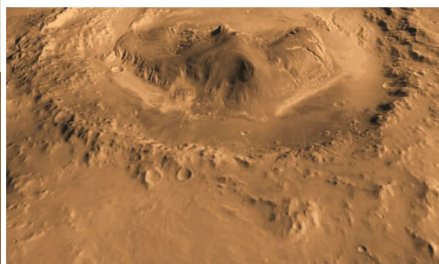
into a warm climate stable enough to get the required amount of erosion done.

Volcanoes might work, too, warming climate by spewing greenhouse gases. The Viking orbiters discovered some of the solar system's largest volcanoes on Mars, but they appeared to have erupted quietly, like the volcanoes on Hawaii, without releasing much gas. Now, in the 3 October issue of *Nature*, planetary geologist Joseph Michalski of the Planetary Science Institute in Tucson, Arizona, and Jacob Bleacher of NASA's Goddard Space Flight Center in Greenbelt, Maryland, report finding the lingering calderas of as many as seven explosive, gas-rich "supervolcanoes" that erupted on early Mars. They might have pumped enough carbon dioxide and other greenhouse gases into the atmosphere to trigger brief spring-

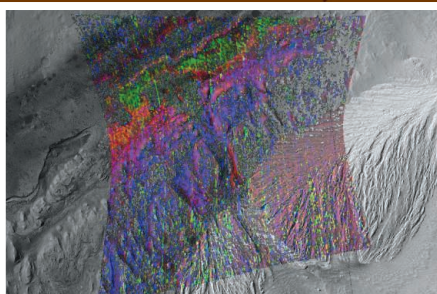
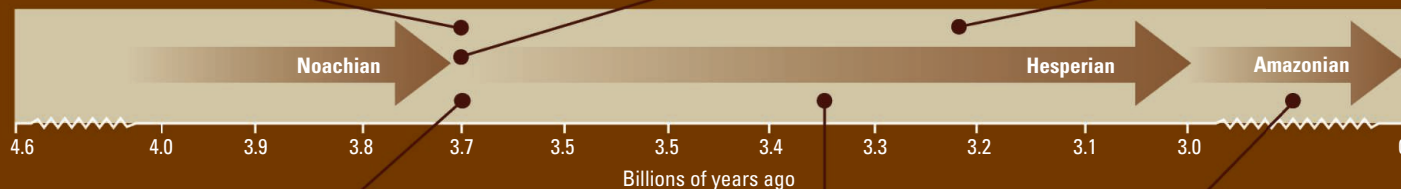
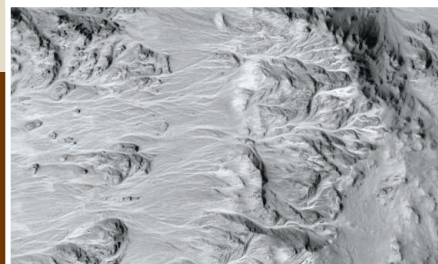
Valley network



Gale crater formation



Alluvial fan



Mount Sharp's spectral clay-sulfate transition



River delta



Amazonian wind deposit on Mount Sharp

times on an otherwise frozen planet.

With a scheme as grand as Head's, there is no shortage of critics. But fluvial geologist Craddock focuses on one very small aspect: the raindrop. The icy highlands scenario has none, but Craddock sees them as essential to explain the observed crater erosion. Only falling raindrops hitting every square centimeter of crater rim and kicking up rock particles can create the observed smooth erosion, he says; meltwater flowing out from glaciers couldn't do that.

But to planetary spectroscopists, who analyze the spectral signatures of minerals from orbit, Head's cold, dry Mars makes sense. A decade ago, the Opportunity rover encountered sulfate minerals that formed when salty, acidic water weathered rock. At first, rover team members attributed them to shallow, salty seas on early Mars. But subsequent rover investigation failed to turn up further signs of standing water. That and mineralogical evidence from the rover persuaded team members that the telltale minerals must have formed underground. Those supposed seas were just ephemeral, briny, and often acidic puddles oozing up from ground water.

Researchers are also rethinking another class of minerals once hailed as supporting a warm, wet early Mars: the clays that

the Mars Reconnaissance Orbiter and Mars Express probes identified in deposits around Mars. Rock weathering produces clays only after prolonged exposure to water. But in the 3 November 2011 issue of *Nature*, Bethany Ehlmann of the California Institute of Technology in Pasadena and several other leading planetary spectroscopists say that there is little need for much standing water on early Mars.

The types of clay detected and the way they have been exposed by impacts "all point to ground water. ... We don't see high-volume flows of [surface] water for long durations," Ehlmann says. In this picture of early Mars, "the proposed warmer and wetter early Mars was largely beneath the surface," the group writes. They conclude that "frozen, arid conditions may have been hallmarks of the surface environment since the early-Noachian period"—that is, the entire known history of Mars.

We just have to go

That's a disappointing verdict for anyone searching the martian surface for signs of ancient life. But NASA planners picked a good spot for determining whether it will stick. Gale crater is one of the lowest spots on

Early wetness, sometimes. Alluvial fans, valley networks, and river deltas all required at least momentary wetness. Wind deposits did not.

the planet, so if water was going to pool anywhere, that was the spot for it. Gale's alluvial fans speak of rushing waters, at least momentarily. Curiosity spotted fine-grained, layered sediments on the crater floor that look for all the world like quiet lake sediments or at least puddle deposits. Stream gullies cut the slopes of Mount Sharp. And the layers in the lower slopes of the central mound preserve a transition from clays to sulfates.

Curiosity has the tools to read the water story in Mount Sharp: how its sediments were laid down, how long water wetted them under what conditions, and—with luck—whether the water ever teemed with life. The tools include cameras with views from the panoramic to the microscopic that can scope out how sediments were deposited. One instrument package will analyze samples drilled from the rock. Another instrument will measure chemical composition from meters away by zapping rocks with its laser. All that could take years of roving, and the story will probably be convoluted. Mars, says planetary spectroscopist Milliken, is likely "no less complicated than Earth."

—RICHARD A. KERR

CREDITS (CLOCKWISE FROM TOP LEFT): ESA/DLR/FU BERLIN/G. NEUKUM; NASA/JPL-CALTECH/ASU/UA; NASA/JPL/UNIVERSITY OF ARIZONA; NASA/JPL-CALTECH/MSS; ESA/DLR/FU BERLIN/G. NEUKUM; NASA/MRO/RALPH MILLIKEN/BROWN UNIVERSITY



CLIMATE CHANGE

Dr. Cool

David Keith has helped usher geoengineering into the mainstream. Actually testing a way to cool the planet is his next big challenge

David Keith was a 26-year-old graduate student in experimental physics when he first heard of geoengineering, the concept of intentionally tinkering with Earth's climate system in order to counteract global warming. It was 1989, and some of his colleagues at the Massachusetts Institute of Technology (MIT) in Cambridge considered the idea distasteful, Keith recalls. Discussing possible experiments was "a de facto taboo" in the field.

That didn't deter Keith, who saw the dearth of interest as a professional opportunity. Three years later, he published his first paper on the topic. Geoengineering needed a "systematic research program," Keith and a co-author concluded after analyzing the few existing studies of possible approaches, including sucking carbon dioxide out of the atmosphere with machines and releasing particles into the sky to block sunlight. Such exotic technologies, they argued, had the "potential to mitigate catastrophic climate change."

Few others, however, paid much attention. And for the next 15 years, discussions of geoengineering drifted between the fringe of academic research and science fiction. Still, as Keith built a career as a specialist on energy and climate issues, he periodically published papers suggesting that scientists needed to take geoengineering seriously.

In the past 5 years, they have. As carbon dioxide continues to build up in the atmosphere, the U.S. National Academies, the United Kingdom's Royal Society, and the American Geophysical Union have all issued calls to explore expanding research into technological fixes. The number of scientists publishing on geoengineering is growing, as are citations of their work by influential groups, such as the Intergovernmental Panel on Climate Change (IPCC).

Keith has ridden geoengineering's shift from the fringe toward the mainstream—and has helped catalyze it. In addition to publish-

Sun blocker. Serious geoengineering research is long past due, says physicist David Keith.

ing influential academic papers, the Canadian scientist has become geoengineering's public face, delivering sold-out lectures and vivid quotes to the media. He's also become something of a power broker, advising one of the world's richest men on climate issues and doling out some \$6 million of Bill Gates's money to convene meetings and spur new research. And 2 years ago, Keith gained a high-profile perch for promoting his views, moving from the University of Calgary in Canada to Harvard University.

Now, Keith wants to bring geoengineering out of the ivory tower and into the stratosphere. He and a partner at Harvard are proposing one of the world's first geoengineering field experiments, using a high-altitude balloon to release sun-blocking vapors into the atmosphere. And this month, Keith is releasing a book, *A Case for Climate Engineering*, in which he argues that "the potential upsides of geoengineering" demand greater research. Such studies "may show that these technologies will not work," he writes. "Yet the sooner we find this out the better."

It's an audacious agenda for a scientist colleagues describe as equal parts thoughtful, unorthodox, and headstrong. And he faces a myriad of obstacles, including a lack of organized government support and fierce opposition from critics—one of whom calls Keith's sun-blocking ideas "barking mad." He's even gotten death threats.

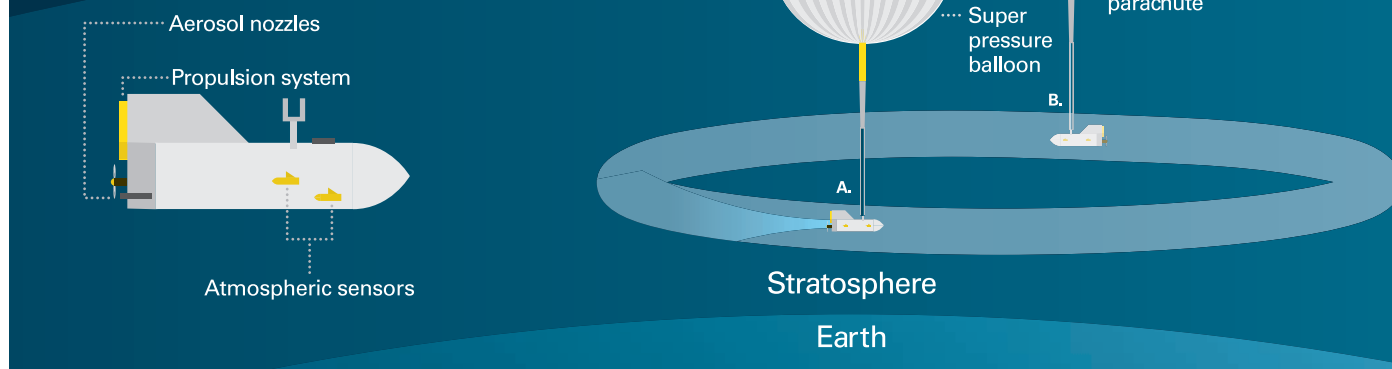
Further complicating matters is Keith's ownership stake in a company that is pursuing a different flavor of geoengineering—sucking carbon dioxide out of the air. That has raised questions about financial conflicts. And some wonder whether Keith has the diplomatic savvy to win over opponents. "David does not suffer fools gladly," says David Layzell, Keith's former boss at the University of Calgary. "Everybody respects him—or is a little terrified of him."

A polymath

Keith grew up in Ottawa, where his father and stepmother, both wildlife biologists, taught him how to stuff birds, enjoy the outdoors, and work with his hands. After earning a physics degree at the University of Toronto, Keith headed to MIT, where "he was an incredible hot shot," recalls Harvard's William Clark, an early mentor.

But Keith was troubled by the military applications of his physics research and instead drifted toward the burgeoning field of climate and energy research. After he earned

Probing the risks of geoengineering



Aiming high. A proposed experiment would use a balloon to release sulfuric acid vapor (A) and then measure its effect on ozone chemistry in successive passes (B).

his doctorate in 1991, his eclectic interests led to an array of jobs over the next decade: policy analysis at Carnegie Mellon University in Pittsburgh, Pennsylvania; climate modeling at the National Center for Atmospheric Research in Boulder, Colorado; building atmospheric instruments at Harvard; and even a stint in environmental ethics at the University of Montana in Missoula. “A lot of rock climbing, too,” Keith says.

Along the way, he published a number of provocative papers, including a 2001 *Science* publication that questioned the potential of wind power to replace fossil fuels, and analyses of hydrogen fuel, natural gas, and the then-controversial concept of carbon capture and storage (CCS): capturing carbon dioxide from industrial smokestacks and pumping it underground.

Geoengineering continued to fascinate him, Keith says, because it provided a new look at humanity’s relationship with nature, which he valued personally as an outdoorsman. Geoengineering “encourages us to rethink some of our root assumptions about the means and ends of climate policy,” he writes in his book.

By 2004, when the University of Calgary recruited Keith from Carnegie Mellon, he was considered a go-to voice on geoengineering, and he began building bridges with business. In 2007, he joined with four top executives in Alberta’s powerful energy industry to write a report touting the potential of CCS to curb carbon emissions. It drew darts from environmentalists, but led to a pledge to invest \$3 billion in CCS technology by the Alberta and Canadian governments, which had requested the study. “I’ve had a deputy minister stop me and say it was amazing what David did on that report,” Layzell says.

Keith’s persuasiveness came with a confidence that could be alienating, however. “David is usually right, and he has a high degree of confidence that he’s right,” says geochemist Ken Caldeira of the Carnegie Institution for Science in Palo Alto, California. And that can be “off-putting to some people,” says Jane Long, an energy scientist at the University of California, Berkeley, who nonetheless commends Keith’s “strong ability to get people to see his point of view, while seeing multiple conflicting points of view.”

In Calgary, Keith’s willingness to speak his mind sometimes complicated his relationships with industry. A few firms refused to partner with the university after Keith challenged Alberta’s efforts to mine its oil sands, for instance. But Keith was becoming an entrepreneur himself, launching Carbon Engineering, a startup aiming to build machines to remove carbon dioxide from the atmosphere, in 2009. One of its investors is Microsoft co-founder Bill Gates, for whom Keith has served as an informal energy adviser since 2006.

The amount of Gates’s investment in the company is undisclosed, but the mogul has also provided roughly \$6 million to the informal Fund for Innovative Climate and Energy Research, managed by Keith and Caldeira. Since 2007, the fund has supported more than a dozen research projects, most on geoengineering. It also helps fund a weeklong summer school on the topic, now in its fifth year, which brings together physical and social scientists. The networking opportunity “shows David’s great value” as a scientist and organizer, says Benjamin Kravitz, a climate modeler at Pacific Northwest National Laboratory in Richland, Washington, whose work is supported by the fund.

Keith’s 2011 move to Harvard, where he

holds appointments in the schools of engineering and government, has brought him close to science and policy heavyweights—and students who go on to become powerful policymakers around the world. It’s also an opportunity to team with some of the nation’s top atmospheric scientists, and ramp up efforts on one of Keith’s priorities, developing rules for governing geoengineering research. And Cambridge offers a bully pulpit for injecting the topic into international discussions. Or, as Keith put it in a 2007 TED talk: “We need a broader debate ... not just a few oddballs like me.”

A low-dose supplement

Keith hopes to jump-start that debate with *A Case for Climate Engineering*. In 112 pages of authoritative prose, he largely eschews figures and technical terms in a bid to reach a lay audience. He begins by casting the climate challenge in stark terms: Past emissions have already committed Earth to substantial warming, he warns; even aggressive emissions cuts—if they ever materialize—can only partly reduce climate risks. But geoengineering techniques could relatively quickly “cut the average rate of global warming in half for the next half-century,” he argues.

In particular, Keith focuses on one technique: releasing sulfuric acid vapor high in the stratosphere, where it would scatter sunlight away from Earth’s surface. The approach mimics the global cooling effect of large volcanic eruptions, which spew sulfates into the stratosphere. But Keith envisions a “slow ramp scenario,” gradually adding sulfur over decades to counteract about one-half the yearly climate change caused by humans. That would allow “ample time” to alter or halt the procedure if there are surprises, he

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notes. And the method could be relatively cheap: A 2010 study he arranged suggested that the cost of starting the plan with aircraft would be, incredibly, a few hundred million dollars—"the price of a Hollywood blockbuster," Keith writes.

Keith highlights the risks. The particles could catalyze chemical destruction of the protective ozone layer, or—at high doses—rob the climate system of crucial energy required to drive precipitation. And even discussing the idea might undermine efforts to transition away from fossil fuels, he concedes, or even prompt international tensions. But some modeling studies, he writes, suggest sun-blocking methods could reduce the harm caused by warming, including heat stress on crops "in the hottest and poorest parts of the world."

Still, many are skeptical. Last month, the IPCC warned that solar geoengineering could "modify the global water cycle," although it didn't specify how much sulfur it might take to cause concern. Other critics are more direct: Efforts to manipulate climate with light-scattering particles are "barking mad," says climate modeler Raymond Pierrehumbert of the University of Chicago in Illinois. One problem, he says, is sun-blocking may do little to reduce overall peak global

to use a balloon release less than a kilogram of sulfuric acid vapor about 20 kilometers above Earth's surface during the fall or spring, when the stratosphere is very still. Chemical sensors aboard the balloon would then measure possible effects on stratospheric ozone. The craft would also repeat the experiment with water vapor, fingered recently by Anderson as a possibly underestimated threat to ozone.

A hazy outlook

Gates, among other tycoons, could fund such an experiment. But Keith is adamant that governments should lead on solar geoengineering research. International oversight agreements and government funding can make "the development of solar geoengineering technologies ... as public and transparent as possible," he writes. Government leadership could also prevent potential conflicts of interest, he argues, by preventing companies from winning monopolies on new technologies and keeping them in the public domain. Keith and Anderson say they will ask the U.S. government to fund their experiment.

So far, however, U.S. agencies have held back from funding geoengineering research, and prospects overseas are dim as well. In 2010, the 197 nations that are members of the Convention on Biological Diversity adopted

A public perch

A publicly funded experiment subject to customary environmental review could avoid such pitfalls, Keith says. But he faces obstacles of his own. One is his stake as president of Carbon Engineering, which some observers say poses a potential conflict of interest because his call for greater investment in geoengineering research could ultimately benefit his own company. Such concerns, in fact, blocked Keith from serving on a current National Research Council panel on geoengineering. "With David straddling this academic-business divide, his company is going to hold him back," Caldeira predicts. "It's impacting his academic career."

Keith sees "a sharp distinction in the role of private enterprise" in the two flavors of geoengineering. Because sun-blocking technologies hold global risks as well as benefits, they are no place for private enterprise, he says. His work in that area involves "open publications and no patenting." In contrast, he argues that firms serve a crucial function in developing air capture methods, which he says pose "local risks" akin to other industry.

Such nuances are often lost in public debate. After one article criticized his proposed experiment, Harvard alumni were "writing to the [university] president ... asking why these maniacs are on your faculty," Anderson says. And then there are the two death threats, apparently from people who believe Keith is part of a government conspiracy. One caller last year was "verbally abusive and drunk," says Keith's assistant Hollie Roberts, prompting a report to the police.

Colleagues, however, appreciate Keith's increasingly public role as advocate. "It's important to have good spokespeople on geoengineering, and Keith is an independent and hyperarticulate one," says Caldeira, of Carnegie. "He's a very deep thinker," Long says. "You may not always agree with him, but you have to hear him out."

For his part, Keith says he's learned from his time in the limelight and is taking greater care in what he says and writes. "It gets under my skin when I am made out to be the rank advocate" for geoengineering, he says. "It hurts." So now he's "trying to be more disciplined about weaving caveats in," so that others can't take his words out of context. And his book tempers boldness with humility. "I myself have concluded that it makes sense to move with deliberate haste towards deployment of geoengineering," Keith writes. "You may well reach a different conclusion. My goal is simply to convince you that it's a hard choice."

—ELI KINTISCH

Geoengineering Milestones

- 1908** Swedish chemist Svante Arrhenius proposes burning fossil fuels to release CO₂ into the atmosphere to warm the planet.
- 1965** In a report to President Lyndon Johnson, scientists suggest spreading particles on the sea surface to reflect sunlight to counteract "deleterious" CO₂ levels in the future.
- 1974** Russian climate modeler Mikhail Budyko proposes burning sulfur in the stratosphere to create a cooling haze.
- 1991** Mount Pinatubo in the Philippines erupts, creating a global layer of sulfuric acid haze that reduces global temperature by about 0.5°C
- 2006** Nobelist Paul Crutzen calls cuts in CO₂ emissions "a pious wish," says geoengineering research is required.
- 2009** The United Kingdom's Royal Society and the American Geophysical Union call for geoengineering research.

temperature increases under many scenarios. And it could even lead to a relatively sudden global temperature spike, he warns, if the effort is interrupted by a war or calamity.

To clarify and quantify such risks, Keith says researchers need to move beyond theoretical debate to actual field experiments. First up, he argues, should be "process studies" that would be too small to have any appreciable impact on climate—studies like the one he and chemist James Anderson of Harvard have now proposed (see graphic, p. 308). The idea is

a resolution that asks governments to oppose "geo-engineering activities that may affect biodiversity." And one U.K.-funded effort, the Stratospheric Particle Injection for Climate Engineering project, had to cancel a 2011 field experiment after it became mired in controversy. Critics complained that researchers hadn't adequately vetted the test, which involved spreading a small quantity of water vapor from a tethered balloon, or worked out how ownership of any new technology might be handled.

LETTERS

edited by Jennifer Sills

China's Rapid Urbanization

BETWEEN 1980 AND 2012, CHINA'S URBANIZATION INCREASED FROM 19.4 TO 52.6% (1). Unfortunately, China's urbanization has developed far ahead of its economic growth. As a consequence, China's urban economic advantages are being offset by the perennial urban

curses of overcrowding, air and water pollution, environmental degradation, contagious disease, and crime (2, 3).

China's rapid urbanization has also resulted in a severe labor shortage in its rural communities. By 2012, 262 million people had migrated to urban areas. The majority of the rural-to-urban migrants are men, who seek higher wages in cities but leave their children, spouses, and aging parents in the villages. The number of rural children left behind increased from 22 million in 2004 to 58 million in 2010, and the women and aging parents left behind have reached more than 47 million and 40 million, respectively (4, 5). These three groups now account for

more than 22% of China's total rural population. This has created societal unrest and psychological development problems for children left behind (4, 5).

The labor shortage in China's rural areas has led to increased uncultivated land and hastened significant changes to traditional agricultural practices, such as reduced tillage, burning of agricultural straw on site, and overuse of chemical fertilizers and pesticides. Overused chemicals have in turn caused widespread water, soil, and air pollution, soil and farmland degradation, and biodiversity loss (6). Of the 16,928 species that are threatened with extinction worldwide, almost 800 are in China; 25% of China's species are endangered, and 233 vertebrate animal species are facing extinction (7). Biodiversity loss has been shown to lead to increased human, animal, and plant diseases. The soaring rate of cancer in cities and the 200 "cancer villages" in China reflect the damage done to the health of its people (8).

China is seeking and implementing innovative solutions that balance economic growth and sustainable development. During the 2012 National People's Congress, the concept of "eco-civilization" was proposed. A year later, creating a green and sustainable urbanization and building an eco-civilization were the key subjects of the 2013 Global Eco-Forum (9). To achieve these ambitious goals, China must refrain from forced urbanization (10) and develop new policies and incentives to retain sufficient workers for its rural communities. By addressing these challenges, China will improve food safety and security, reduce dependency on chemical fertil-

izers and pesticides, improve air and water quality, and help to protect wild plant and animal populations. At the same time, China needs to address the urban issues of reducing air pollution and providing clean water, safe neighborhoods, and efficient infrastructure—the basics of city living (11).

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Gain-of-Function Research: Unproven Technique

THE LETTER "GAIN-OF-FUNCTION EXPERIMENTS ON H7N9" (R. A. M. Fouchier *et al.*, 9 August, p. 612; published online 7 August) is based on unproven assumptions from previous H5N1 studies and on less-than-critical examination of the basic biology of influenza viruses (1). Furthermore, there is no scientific basis for the claim that gain-of-function (GOF) research on H7N9 may lead to development of more effective vaccines. There is a need to advance influenza vaccine development, but GOF experiments are

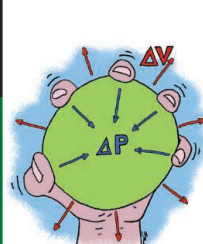
Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.



Ultrafine for
extra strength

320



Sound control
strategies

323

unlikely to provide the required insights.

Influenza vaccines have been manufactured for many decades based on the isolation of a virus with a specific pandemic potential or seasonal prevalence. These isolates need to be propagated in eggs or, more recently, in cell cultures. Once obtained in sufficient quantities, vaccines are prepared either by inactivating the whole virus particle or isolating a particular fragment. Recently, a vaccine was produced by cloning viral hemagglutinin (2). It has so far been necessary to produce a new vaccine to protect against every influenza virus suspected of pandemic or seasonal threat, irrespective of the structure of viral hemagglutinin or detected mutations in its amino acid sequence.

Fundamental scientific studies have previously established that influenza viruses have high error rates in their viral polymerase and variation in their propensity to infect many avian and mammalian species. Furthermore, evolutionary pressures result in multiple reassortment and mutational events that follow no clear pathway and are impossible to predict or associate with a specific outcome in any population (3). Aside from its biosecurity and biosafety risks, GOF research is not based on scientific grounds, but rather on highly speculative assertions. Experience to date with H7N9 viruses in regard to neuraminidase inhibitor activity or the presence of certain hemagglutinin motifs are not novel findings (4). The previous GOF studies on H5N1 contributed little to the development of new vaccines or therapeutic measures. The current proposal is based on self-serving advocacy rather than a scientific determination.

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Gain-of-Function Research: Unknown Risks

WE WERE ASTONISHED BY THE RECENT LETTER by R. A. M. Fouchier *et al.* ("Gain-of-function experiments on H7N9," 9 August, p. 612; published online 7 August) and the simultaneous publication in *Nature* (1). We find it odd that an H7N9 influenza A research manifesto signed by a number of flu virologists should be aired so prominently, coming as it does from a distinguished but unrepresentative group. The Letters were disavowed within 24 hours by opinions from two influential Chinese influenza scientists (2, 3). Publishing research manifestos is de rigueur for projects that require setting up colossal infrastructure, such as the hunt for the Higgs boson. However, the proposed agenda for H7N9 research is obvious to any experienced infectious disease researcher. Indeed, H7N9 in the Fouchier *et al.* Letter could be replaced by MERS-coronavirus or any newly discovered virus that poses a perceived threat.

More egregiously, Fouchier *et al.* try to make the point that gain-of-function (GOF) experiments are now a standard part of a virology research agenda. This astonishing assertion is simply not true. There are some



very strong arguments to the contrary; for example, avian influenza GOF experiments cannot prove their point because the deliberate infection of humans is impossible. They can infer and suggest, but no more. Apart from the implausibility of predicting what nature might turn up (4, 5), inferences are of limited value to a health minister. Solid data are what counts. Questions have been raised about the statistical robustness of the findings from the key H5N1 GOF studies given the small numbers of animals used (6).

Even though there are substantial risks involved in GOF research, surprisingly, no independent risk-benefit assessment has been undertaken, which is deeply troubling given the magnitude of the risk—a man-made flu pandemic. Given that scientists still have not reached a consensus regarding GOF research, the benefits cannot be currently quantified. By contrast, the risks are real and can and should be quantified. A compelling argument can be made that GOF work should be frozen until we have a comprehensive risk assessment.

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CORRECTIONS AND CLARIFICATIONS

Editors' Choice: "Beating fluorescent background" by P. Szurmi (13 September, p. 1153). The luminescent lifetime of the nanoparticles is 5 to 13 μ s, not 5 to 13 ms. The HTML and PDF versions online have been corrected.

News & Analysis: "Researchers wary as DOE bids to build sixth U.S. climate model" by E. Kintisch (13 September, p. 1160). The article stated that the Department of Energy provides \$6.2 million this year for the Community Earth System Model (CESM). To clarify, the \$6.2 million supports the National Center for Atmospheric Research but does not represent the Department of Energy's total support for CESM. The HTML and PDF versions online have been corrected.

Reports: "Positive feedback between PU.1 and the cell cycle controls myeloid differentiation" by H. Y. Kueh *et al.* (9 August, p. 670; published online 18 July 2013). The second author's name was misspelled. It should be Ameya Champhekar. The HTML and PDF versions online have been corrected.

GENOMICS

The Godfather, Part II

Sophia Roosth

Craig Venter is a singular individual who aspires to be universal. In *Life at the Speed of Light*, he fantasizes this: “since my own genome was sequenced, my software has been broadcast in the form of electromagnetic waves, carrying my genetic information far beyond Earth, as they ripple out into space. Borne upon those waves, my life now moves at the speed of light.” He wonders whether an alien civilization might understand and act upon his genome’s broadcast “instructions.”

No doubt, the Earthbound Venter already wears many hats: scientist (racing the National Institutes of Health to sequence the human genome), sailor (captaining *Sorcerer II* to sequence the Sargasso Sea), and biotycoon (aggressively commercializing his research). Whereas Venter’s previous book (1) traced his career and personal life, he now offers a 21st-century response to the question physicist Erwin Schrödinger posed at Trinity College, Dublin, in 1943: “What Is Life?” Seventy years later, Venter strode across the same stage to tackle the same question. Like his predecessor (2), he has produced a book based on his lecture, and he opens by comparing his thinking to Schrödinger’s. The book roughly divides into three sections: historical, biographical, and speculative. Venter first looks back to trace the past 60 years of molecular biology, identifying thematic precursors to synthetic biology (e.g., Jacques Loeb’s “engineering ideal,” Friedrich Wöhler’s “proof by synthesis”). Later, he looks ahead to imagine synthetic genomics’s future. In between past and prospective, Venter situates himself as a pivot. He is at once inheritor of molecular biology’s prior triumphs and the wellspring of its bright future.

The story really gets under way when Venter narrates his involvement in the past 30 years of genomics (although he skims over the Human Genome Project, which he detailed in his earlier book). He compellingly depicts his diverse research as a concerted effort to shuttle biology between the material and digital

worlds. Through separate chapters on four key experiments, he describes sequencing the genomes of various bacteria and archaea. Such thinking galvanized Venter’s later efforts (starting with synthesizing bacteriophage phi X 174) to move not only from digital to material but also in the other direction. The book is at its best when readers are allowed a glimpse into the J. Craig Venter Institute’s (JCVI’s) synthesis of *Mycoplasma*, beginning with synthesizing the *M. genitalium* genome. JCVI scientists next transplanted a *M. mycoides* genome into a recipient *M. capricolum* cell, demonstrating that the cells were induced to grow and divide as *M. mycoides* cells—a spectacular technical feat.

This project culminated in JCVI’s pièce de résistance: sequencing the *M. mycoides* genome, synthesizing and assembling it in a yeast cell, and transplanting it into *M. capricolum* cells to produce “the first living self-replicating species to have a computer as a parent.” The memoir’s dramatic climax is replete with 4 a.m. text messages; hands shaking with anticipation as they grip incubator doors; and (spoiler alert!) scientists reveling in success, popping bottles of champagne, exchanging birthday balloons, and speaking at clamorous press conferences. This research will be familiar to readers of *Science* (in whose

pages the results were published). Still, the day-to-day setbacks that plagued researchers as they “booted up” the cell and their thrill at finding a single blue colony on a Petri dish provide a gripping tale and welcome antidote to dry materials and methods sections that make such sagas feel disembodied and inevitable. Throughout, Venter’s self-fashioning is careful: He savors his perceived iconoclasm (for example, the first thing future collaborator Ham Smith said to Venter is “I thought you were supposed to have horns”). He believes

himself righteous yet embattled, as when his choices to sequence genome fragments and patent expressed sequence tags were met by fellow scientists with reactions ranging from distaste to fury.

Venter’s “synthetic cell” and the breathless press it garnered—which posed Venter as both god and parent to synthetic life—have been criticized by some who claim the cell isn’t truly “synthetic”: the genome wasn’t designed but sequenced from extant *M. mycoides*. Further, it was inserted into a recipient cell and thus can’t be made “from scratch.” Venter defends himself against



detractors by arguing that such disputes misunderstand what both “synthetic” and “life” mean. He dispenses with his critics by privileging the genome as the definition of life, noting that “creating” “synthetic” “life” affected his thinking about life itself: “Our experiments did not leave much room to support” proponents of “a ‘vital force’ that [distinguishes] the animate from the inanimate.”

And this is Venter’s great white whale: Like Schrödinger before him, he has vitalism squarely in his crosshairs. Why, he wonders, has it been resilient enough to survive so many experimental assaults? He bemoans a strain of 21st-century vitalism that “manifests itself in the guise of shifting emphasis away from DNA to an ‘emergent’ property of the cell that is somehow greater than the sum of its molecular parts.” In his view, any understanding of biology that does not privilege DNA as *primum movens*—including the sin of “attribut[ing] unmeasurable properties to the cell cytoplasm”—is tantamount to neovitalism. His synthetic cell, in his eyes, hammered the final nail in vitalism’s coffin. Not bad for someone in the business of creating life.

Life at the Speed of Light
From the Double Helix to
the Dawn of Digital Life

by J. Craig Venter
Viking, New York, 2013.
236 pp. \$26.95, C\$28.50.
ISBN 9780670025404.
Little, Brown, London. £20.
ISBN 9781408705247. Paper,
£14. ISBN 9781408705254.

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In the book's denouement, Venter imagines futures when biology can shuttle seamlessly between the material and the informatic. Drawing examples from *Star Trek* and *Doctor Who*, he speculates that DNA sequencing and synthesis are paired technologies for dematerializing, digitizing, and subsequently rematerializing life. He's confident that soon "we will be able [to] send a robotically controlled genome-sequencing unit in a probe to other planets to read the DNA sequence of any alien microbe life that may be there." If such a sequence is beamed back from Mars, "we should be able to reconstruct the genome. The synthetic version of the Martian genome could then be used to recreate Martian life."

Although fantastic, such scenarios proffer one answer to Schrödinger's question. What was life in 1943, what is it in 2013, and what will it become next? Despite staggering developments in molecular biology since the 1940s, I'm struck by how little has changed. If Venter is to be believed, life itself has been recreated, yet the same hoary debates are still being aired: mechanism versus vitalism, form versus substance, experimental deduction versus proof by synthesis. Life, it seems, moves more slowly than Venter supposes.

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THE INTERNET

The Cyber-Espionage X-Files

Laura DeNardis

Former U.S. Secretary of State Hillary Clinton once delivered a soaring speech on Internet freedom urging American media corporations to challenge "foreign governments' demands for censorship and surveillance." Three years later, Internet-freedom advocates experienced the cognitive dissonance of juxtaposing this rhetoric with revelations about the U.S. National

Security Agency's (NSA's) expansive digital surveillance practices. These disclosures came as no surprise to those who have read Ron Deibert's *Black Code*.

Although the Internet's exosphere of content and applications is visible to users, the majority of the network's technical and material architecture lies deeply concealed beneath this surface. The structure and governance of the Internet's underlying infrastructure—comprising code, hardware, protocols, switches, and other virtual and physical resources—is hardly neutral but rather constrains behavior through technical design, direct governance, and private contractual agreements. The multivariate metaphor "black code" refers to this hidden nature of cyberspace infrastructure and also to the clandestine sphere of nation-state intelligence gathering and warfare in cyberspace (invoking Black Ops) and more nefarious online criminal practices (invoking Black Hat hacking).

Many readers will find *Black Code* both illuminating and terrifying. State power over the Internet is escalating, with the exact same technologies that provide unprecedented opportunities for free expression being used to enact surveillance and censorship of citizens by authoritarian governments and liberal democracies alike. In a book researched and penned prior to Edward Snowden's whistleblowing on NSA surveillance, Deibert (a political scientist at the University of Toronto) cites a former NSA employee who estimated that "billions" of phone calls and "voluminous" quantities of e-mails are electronically processed every day.

Government surveillance does not happen *sui generis* but requires cooperation from the private companies—social media platforms, search engines, and transactional sites—that serve as information intermediaries. Big data gathered around routine Internet use through these private intermediaries fuel the online advertising business models enabling free and unprecedented access to knowledge. But collecting these data (including metadata such as geographical location, phone number, and unique technical identifiers) creates unprecedented challenges to individual privacy and opportunities for surveillance. As Deibert graphically portrays private industry data capture, "Like a giant python that has consumed a rat, Facebook captures, swallows, and slowly digests its users."

One of *Black Code*'s contributions is to illumine the privatized political architecture

of the Internet's core technologies. Deibert once toured a Toronto Internet Exchange Point, a shared switching facility at which networks conjoin to collectively compose the global Internet. A tour guide's response to his query about "hundreds of what appeared to be randomly distributed red tags attached to the equipment" was "Oh, those are the wiretaps."

Deibert also unearths the subterranean battles at the Internet's core via his account of the investigative work of the University of Toronto's Citizen Lab, which he founded in the spring of 2001 to "lift the lid on the Internet" and research global security issues in cyberspace. Using a combination of technical forensic analysis and social science research methods, Citizen Lab exposed a disturbing world of digital subterfuge, including "Ghost-Net," a global cyber-espionage network that had compromised computers operated by ministries of foreign affairs, major news outlets, embassies, and numerous global institutions. The Dalai Lama's computers having been infiltrated, his administration granted Citizen Lab unrestricted access to them. Citizen Lab analysts infiltrated a cyber-espionage operation for months, discovering that the attackers used the freely available open-source cyber-intrusion tool Ghost RAT.

The book also conveys examples of the Internet's vulnerability to inadvertent problems, such as when Internet access in the nation state of Georgia "went dark" for 12 hours after an elderly woman with a shovel accidentally severed a critical fiber-optic cable. Basic societal structures of financial flows, energy infrastructures, social interactions, and commerce depend on the Internet, an infrastructure whose technical architecture and governance are in constant flux.

Black Code ties together usually disparate subject matter—big data, new business models based on online advertising, cyber-crime, infrastructure vulnerability, and geopolitical power struggles—into a coherent exposé of what is at stake in how the Internet is designed, governed, and infiltrated. In it, Deibert uncovers more problems than solutions (which he addresses elsewhere), but this is a refreshing change from prevailing narratives about social media-driven democratic revolutions. In reality, the Internet's architecture is now ground zero for geopolitical conflict, rising state power, and the future of what counts as basic civil liberties in the digital era.

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Black Code

Inside the Battle for Cyberspace

by Ronald J. Deibert

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Probiotics: Finding the Right Regulatory Balance

D. E. Hoffmann,^{1*} C. M. Fraser,² F. B. Palumbo,³ J. Ravel,² K. Rothenberg,¹ V. Rowthorn,¹ J. Schwartz¹

Some products marketed as drugs should be excused from Phase I trials, but safety and efficacy claims for dietary supplements should be more tightly regulated.

Initial findings of the Human Microbiome Project (HMP), funded by the U.S. National Institutes of Health (NIH), raise important questions about the role and variation of microorganisms within individuals and across populations (1). One related area of growing research and commercial interest is the development and use of probiotics, substances containing live microorganisms that have a beneficial effect when taken in sufficient quantities (2) and “designed to intentionally manipulate microbiome and host properties” (3). We offer observations about the regulatory process for probiotics and potential areas for reform.

Most probiotics—consumed for centuries as yogurts and fermented milks—are sold as foods or dietary supplements. In recent years, the variety of probiotic foods on supermarket shelves has expanded, and probiotic dietary supplements are being aggressively marketed in retail stores and on the Internet. Although no probiotic has been approved for therapeutic purposes, some are undergoing clinical trials and may soon be marketed as biologics or other drugs (4). There is evidence of the potential benefit of some strains and species of probiotics for a variety of indications (5).

In addition to promoting the development of new clinical therapies, the HMP is likely to increase the number of probiotic foods and dietary supplements for consumers, as well as the claims made about them (6). Consumer demand for these products is growing, in large part because of their health and wellness claims (7). Although some of these claims may have merit, others do not (8, 9).

Under an NIH-HMP funded ELSI (Ethical, Legal, and Social Issues) study, we convened a group (10) to examine whether the current U.S. regulatory framework for probiotics: (i) adequately addresses issues of safety and effectiveness; (ii) provides sufficient information to consumers to make informed

choices; and (iii) sufficiently allows for, or at least does not discourage, research on potential therapeutic benefits (11).

The U.S. Food and Drug Administration (FDA) has no definition of probiotics and regulates them based on whether they fall into one of the existing regulated product categories (e.g., drugs, biologics, foods) (12). Yet, regulatory approaches for other products may not cover some unique features of probiotics. Probiotics are live, dynamic organisms, likely to lose viability and degrade over time. Their research and manufacturing involve a greater number of variables than does research with many other substances. These include the effect of the environment on the viability of the probiotic and interaction with the human genome and microbiome. Without stringent manufacturing procedures and quality controls, specific probiotics may lose the properties that once formed their isolation and selection criteria (13). Animal models are limited because of differences between human and animal microbiomes and immune systems. Many probiotics are consumed by individuals on a daily basis as foods, which makes dosing for therapeutic purposes challenging.

An example of the questionable fit between traditional regulatory concerns and probiotics is 2010 FDA guidance for early clinical trials using live biotherapeutic products (LBP) (14). Without using the word “probiotic,” the guidance appears to incorporate probiotics into this category. But the requirements are not entirely relevant for probiotics, in that they require a summary of the phenotype or genotype of the strain with specific “attention to biological activity or genetic loci that may indicate activity or potency” (14). It is difficult to pinpoint genetic loci for probiotics, especially in early clinical trials. LBP product characterization standards may be inappropriate for probiotics, as safety and effectiveness may depend on both the product and the microbiome of the consumer.

Changing FDA’s Regulatory Framework

Although probiotics have some distinctive characteristics, they arguably are not unique enough to warrant their own regulatory path-

way, largely because probiotic products are so varied and may be marketed as foods, dietary supplements, medical foods, foods for special dietary use, or drugs. In addition to changes regarding characterization, two modifications to the regulatory framework could improve how probiotics are addressed by FDA.

Abbreviated application process. Under the current regulatory framework, if the intent of a study is to substantiate a drug claim (that a substance can diagnose, cure, mitigate, treat, or prevent disease), researchers must submit an Investigational New Drug Application (IND) to FDA. The IND may include results of pharmacologic and toxicity studies; chemistry, manufacturing and controls data; and a clinical plan. It generally includes three phases of human studies for development of the new product. In some cases, high costs of the IND have been an obstacle to research.

Probiotic products that include drug claims generally should be subject to the same rigorous requirements as other products making drug claims, including adequate and well-controlled investigations supporting such claims. But, under limited circumstances, we recommend an abbreviated IND (AIND) process for some probiotic products that would allow them to bypass phase 1 clinical safety studies (11). Eligible for this process would be probiotic foods, dietary supplements, and dietary ingredients for which there is adequate evidence of safety in the target population; approved food additives; and substances generally recognized as safe (GRAS).

The AIND probiotic would be required to be studied in the same dose (or amount) and delivery system as the probiotic previously deemed safe. Under the AIND process, if the sponsor wished to conduct a study to support a therapeutic benefit for an at-risk population, FDA would need to determine whether the available safety information is suitable for this target population. The AIND would provide an abbreviated pathway for some probiotic foods and dietary supplements to make drug claims, albeit by moving them into the drug category.

Regulating claims. FDA regulates advertising claims for prescription drugs and label-

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PROBIOTIC PRODUCT CLAIMS ALLOWED BY HEALTH CANADA PROBIOTICS MONOGRAPH

Eligible General Claims	<i>Lactobacillus johnsonii</i> La1	<i>L. johnsonii</i> Lj1	<i>L. johnsonii</i> NCC 533	<i>L. rhamnosus</i> GG	<i>Saccharomyces boulardii</i>
Probiotic to benefit health and/or to confer a health benefit.	●	●	●	●	●
Provides live microorganisms to benefit health and/or to confer a health benefit.	●	●	●	●	●
Probiotic that forms part of a natural healthy gut flora.	●	●	●	●	
Provides live microorganisms that form part of a natural healthy gut flora.	●	●	●	●	
Probiotic that contributes to a natural healthy gut flora.	●	●	●	●	
Provides live microorganisms that contribute to a natural healthy gut flora.	●	●	●	●	
Eligible Specific Claims					
An adjunct to physician-supervised antibiotic therapy in patients with <i>Helicobacter pylori</i> infections.	●	●	●		
Helps to manage acute infectious diarrhea.				●	
Helps to manage antibiotic-associated diarrhea.				●	
Helps to reduce the risk of antibiotic-associated diarrhea.				●	●

ing claims for essentially all FDA-regulated products, including prescription and over the counter (OTC) drugs, dietary supplements, and food (15). FDA regulation of claims differs according to which category a product falls within. Drug claims or health claims, i.e., claims of a reduction of risk of disease, require FDA approval before marketing. Foods and dietary supplements may make claims, without premarket approval, about the role of a nutrient or dietary ingredient intended to affect normal structure or function of the body in humans. The manufacturer is responsible for ensuring the accuracy and truthfulness of these claims; however, lack of prior approval presents an opportunity for misleading and unsubstantiated claims.

Structure-function claims may be difficult to substantiate. For example, unique to probiotics are claims that they maintain or promote a healthy “balance” of microorganisms in the body. This balance concept is not part of the disease-focused paradigm that has governed regulation of health-related products in the United States, in large part because, although some have suggested conceptual approaches to measurement of balance [e.g., (16)], there is a paucity of measurable outcomes to determine balance and whether it is beneficial.

Problematic advertising claims including unsubstantiated structure-function and unapproved drug or health claims are due to lack of agency enforcement resources, the difficulty of policing advertising on the Internet, and to the lure of profit by potential manufacturers. In addition to greater enforcement, we recommend that FDA establish a monograph for probiotic foods and dietary supplements that could be modeled on that adopted in Canada for natural health products or FDA’s monographs for OTC drugs (11).

The benefits of the monograph approach are twofold. Because claims permitted by a monograph would be evidence-based, unsubstantiated structure-function claims would likely be reduced. Further, mono-

graph-approved drug or health claims could be made without subjecting the product to the current and more costly IND process.

Most probiotic products that would be considered dietary supplements in the United States are regulated in Canada as natural health products and fall under probiotics and live microorganisms monographs (17) that cover acceptable ingredients, doses, formulations, and quality specifications, and specify the claims that can be made about these products. Under Health Canada’s probiotics monograph, all probiotic natural health products require premarket assessment and licensing and must be supported by evidence of strain-specific safety and efficacy under recommended conditions of use. Compliance with the monograph requirements leads to expedited review of the application for marketing the product. The monograph allows specific and general claims for strains that meet all additional requirements [see table, based on (17)]. Natural health products are not limited to these claims, but additional evidence supporting safety and efficacy is required for claims not specified by the monographs.

Unlike Canada’s monograph, FDA’s OTC drug monographs do not require pre-market approval. FDA could create probiotics monographs for strains it believes are GRAS and effective for a particular benefit and could use expert panels as it did in developing OTC drug monographs. Similar to most FDA OTC drug monographs, a probiotics monograph would list, among other things, active ingredients, acceptable product claims, labeling, and dosages. Ideally, a monograph would reduce the number of unsubstantiated probiotic claims and thereby help consumers make more informed decisions.

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- The Federal Trade Commission (FTC) regulates advertising claims for OTC drugs, foods, dietary supplements, medical devices, and cosmetics.
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NEUROSCIENCE

Sleep It Out

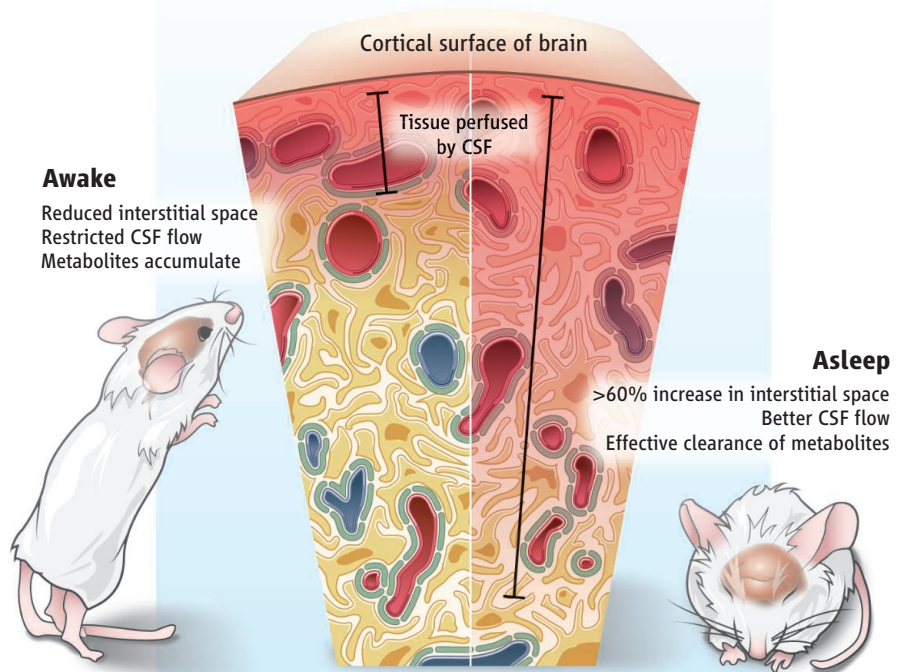
Suzana Herculano-Houzel

We know from personal experience that sleep is not just another brain state but a basic requirement for normal brain function while we are awake. Mental fatigue, poor decision-making, impaired learning, and a heightened risk of migraine and epileptic attacks ensue when we are sleep deprived—and chronic and complete insomnia ultimately lead to death in humans, rats, and flies alike (1). Why does normal brain function deteriorate with prolonged waking and require sleep to be restored? On page 373 in this issue, Xie *et al.* (2) report that during sleep, waste products of brain metabolism are removed from the interstitial space among brain cells where they accumulate. Sleep, therefore, might be required for potentially toxic metabolites—the very results of a working brain—to be cleared from the tissue.

The interstitial space between cells in a tissue is an underappreciated component of the nervous system, considering that it amounts to ~20% of the total volume of the living, anesthetized brain (3)—which is the state in which most studies of brain physiology are made. In the absence of a lymphatic system in the brain, the interstitial space performs its duties, removing waste products that brain cells secrete. These metabolites are flushed away through the interstitial space by the flow of cerebrospinal fluid (CSF), which is filtered from blood and pumped into the brain by the choroid plexus at the ventricles, seeps through the interstitial space among brain cells, and is eventually pumped back into the blood stream at the meninges that surround the brain.

By applying real-time iontophoresis (to observe fluid flow), Xie *et al.* estimate that the interstitial space in the waking mouse brain is only 14% of brain volume, but increases by over 60% in natural sleep or anesthesia to 23% of the brain volume (see the figure). This might appear to be a relatively modest increase in interstitial space, but has remarkable consequences for the flow of CSF and the substances carried in it. Using fluorescent tracers injected into the CSF and visualized by two-photon microscopy of the exposed

Change in the brain's extracellular space between sleep and waking states may drive the clearance of metabolites and toxins.



Volume variation. The extracellular (interstitial) space in the cortex of the mouse brain, through which cerebral spinal fluid moves, increases from 14% in the awake animal to 23% in the sleeping animal, an increase that allows the faster clearance of metabolic waste products and toxins. Therapeutics could potentially exploit this dynamic to clear factors associated with conditions such as epilepsy, migraines, and insomnia.

mouse brain, the authors find that in waking, CSF flow is restricted to the brain surface—but expands deep into the tissue during both natural sleep and anesthesia. The consequence is remarkable: The flow of CSF through the interstitial space is reduced during waking to only 5% of the flow found in sleep. Moreover, both the constriction of interstitial space and the restriction of CSF flow through it observed in natural waking can be mimicked by the application of a noradrenergic agonist to the surface of the sleeping brain. This molecule simulates the release of noradrenaline, which only occurs in the brain during waking. Noradrenaline modulates neuronal activity, which can alter the volume of the interstitial space (4) by causing cells to swell in the presence of the elevated extracellular K^+ concentration that results from neuronal activity (3). The state-dependent constriction of this space might thus be a direct consequence of the molecules associated with waking.

The striking increase in CSF flow through the interstitial space during sleep results in

much more efficient clearance from the brain of metabolites, such as β -amyloid ($A\beta$), a peptide that accumulates during waking and that has been implicated in the progression of Alzheimer's disease. Xie *et al.* found that both radiolabeled $A\beta$ and another inert tracer injected into the cerebral cortex are cleared two times as fast from the brain in sleeping compared to waking mice.

Xie *et al.* propose that the restorative function of sleep may be due to switching of the brain into a state that facilitates the clearance of degradation products of neural activity that accumulate during wakefulness. But going one step further, it is possible that the very accumulation of metabolites during waking, forced by the constricted interstitial space, drives the switch to sleep state. This then allows the clearance of metabolites and prepares the brain for a new bout of waking, in an obligatory, self-regulating, and never-ending alternation of brain states.

A similar idea was behind the proposition that adenosine, a metabolite of both neuronal and glial activity, serves as a sleep-induc-

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ing molecule that underlies the homeostatic drive for sleep (5). The concentration of adenosine in the brain increases during waking, accumulates even more with sleep deprivation, and decreases rapidly during sleep (6), even during only brief intrusions of sleep into forcefully prolonged wakefulness (7). The findings by Xie *et al.* offer a testable explanation for this fast restorative effect of intrusive sleep—the dissipation of sleep pressure as metabolites, such as adenosine, are rapidly swept away.

The observations of Xie *et al.* should spark interest in the state-dependent clearance of waste metabolites, as the wake-shrinking interstitial space presents a good place to look for factors whose accumulation during waking heightens sensitivity to migraines and epileptic seizures, and worsens other conditions associated with insomnia. The development of drugs that facilitate clearance of waste products during waking is also a new possibility.

The newfound interest in the interstitial space of the brain and how it relates not only to metabolite clearance but also to brain states might also help resolve another sleep-related conundrum: the relationship between the number of hours of sleep characteristic of each species and their brain size (8). Sleep is universal among vertebrates (9) and has been found in invertebrates (9, 10). The total number of hours of daily sleep varies from as much as 20 hours in bats to as little as 3 to 4 hours in giraffes and elephants (8, 11)—and there is currently no reasonable physiological hypothesis to explain this variation (11). Because CSF perfusion of the interstitial space is limited to the surface of the brain during waking, and brain volume increases faster than brain surface area [even with the folding of the cortical surface (12)], larger brains should have a relatively larger volume of interstitial space to “buffer” the accumulation of sleep-driving molecules, and thus might be able to withstand much longer

periods of waking before the inevitable switch to the waste-clearing state of sleep occurs. If only neuroscientists could easily bring live, large-brained animals to the lab.

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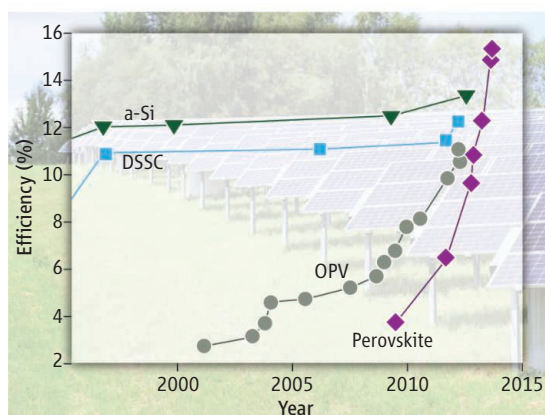
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APPLIED PHYSICS

Perovskite-Based Solar Cells

Gary Hodes

Photovoltaic (PV) cells that convert sunlight directly into electricity are becoming increasingly important in the world's renewable energy mix. The cumulative world PV installations reached around 100 GW_p (gigawatts) (1) by the end of 2012. Some 85% use crystalline Si, with the rest being polycrystalline thin film cells, mostly cadmium telluride/cadmium sulfide ones. Thin-film cells tend to be cheaper to make with a shorter energy payback time. However, they do have the disadvantage, one that may become crucial when considering the terawatt range, that most of them contain rare elements like tellurium (as rare as gold), indium, and gallium. A newcomer to the PV field (2) has rapidly reached conversion efficiencies of more than 15% (see the figure). Based on organic-inorganic perovskite-structured semiconductors, the most common of which is the triiodide (CH₃NH₃PbI₃), these perovskites tend to have high charge-carrier



mobilities (3, 4). High mobility is important because, together with high charge carrier lifetimes, it means that the light-generated electrons and holes can move large enough distances to be extracted as current, instead of losing their energy as heat within the cell. On pages 344 and 341 of this issue, Xing *et al.* (5) and Stranks *et al.* (6) use time-resolved transient absorption and photoluminescence to show that the effective diffusion lengths are indeed relatively large in CH₃NH₃PbI₃, about 100 nm for both electrons and holes—a high value for a semiconductor formed from solution at low temperature.

Organic-inorganic hybrid semiconductors may provide the basis for the next generation of thin-film solar cells.

Onward and upward. Comparing the rate of increase in perovskite solar cell efficiencies (purple lines and markers) with leading third-generation (i.e., relatively new) solar cells and with amorphous Si (a-Si), green; dye sensitized, blue; organic, gray. The first two perovskite cells (2009 and 2011) refer to liquid junction cells, which were not stable but were important in initiating the subsequent solid-state cells. The last three cell types are taken from www.nrel.gov/ncpv/images/efficiency_chart.jpg.

Another important consideration for these perovskites is that they are deposited by low-temperature solution methods (typically spin-coating). The low energy and ease of deposition is of obvious importance for eventual manufacturing of the cells. It also greatly emphasizes the importance of the diffusion lengths described in these two papers for CH₃NH₃PbI₃. For those working on more conventional semiconductor films, the reported diffusion lengths of 100 nm may not appear to be special. However, low-temperature (below 100°C) solution-processed films tend to have considerably smaller diffusion lengths. Stranks *et al.* had previously described nanostructured cells using CH₃NH₃Pb(I,Cl)₃ (essentially the iodide with a small amount of chloride) (7) and demonstrated a thin-film solar cell (not nanostructured) with an

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11.4% conversion efficiency (8) [and, more recently, 15.4% using vacuum evaporation of the perovskite, which results in more uniform films (9)]. Because the perovskite film thickness was about 500 to 600 nm, this implies that the electron and hole diffusion lengths were at least of this order. Stranks *et al.* measured values of the diffusion length exceeding 1 μm for the mixed perovskite, an order of magnitude greater than the already impressively high value of around 100 nm for the pure iodide. Stranks *et al.* also showed that the electron and hole lifetimes in the mixed perovskite is longer than in the pure iodide, which can explain the longer diffusion length in the former. But they note that the reason the apparently small amount of chlorine has such a pronounced effect is an open question.

Although the large diffusion lengths can explain the high quantum efficiencies and photocurrents obtained with these materials, there is another characteristic of these cells that is no less exciting—the very high values of open-circuit voltages (V_{OC}) typically obtained. For $\text{CH}_3\text{NH}_3\text{PbI}_3$, V_{OC} typically approaches 1 V, while for $\text{CH}_3\text{NH}_3\text{Pb}(\text{I},\text{Cl})$, $V_{\text{OC}} > 1.1$ V has been reported (7). Because the band gaps (E_g) of both these semiconductors are 1.55 eV, this results in much higher V_{OC} -to- E_g ratios than usually observed for similar third-generation cells (10). With higher band-gap perovskites, V_{OC} up to 1.3 V has been demonstrated (11).

There are three main considerations that will affect the outlook for these cells. First is the energy conversion efficiency. But with an efficiency of 15.4% after only several years work and no reason to believe that this is close to the limit, that aspect is in good shape.

Second is cost, which is more complicated because it includes energy cost (and energy payback time), as well as availability of the raw materials. The low temperature solution methods used translate into overall lower energy requirements in the cell manufacture. There are no rare elements involved (the gold back contact can be replaced with a much cheaper contact material). The use of lead will worry some. While there are other possible replacements (such as tin), it should not be a major issue even if lead is needed in commercial cells. To put things in perspective, for a production capacity of 1000 GW per year, less than 10,000 tons of lead would be needed. Compare this with the 4 million tons per year of lead used for lead-acid batteries.

The third aspect is stability. There are a few studies on storage lifetime but only one to date on an operating cell (under illumination at maximum power) for a sealed cell at 45°C (12). The study showed a decrease in efficiency of less than 20% after 500 hours. This is actually very encouraging. We just have to look at the advances made in organic cell stability over the years to realize that there is cause for optimism that these cells can be made commercially viable.

There is an enormous effort now under way in these cells. It is by no means unrealistic to expect development and production of these cells, if initially on a small scale, in a relatively small number of years. The fact that these semiconductors can be made so simply and yet with such good crystalline and electronic properties (something that was recognized previously, but clearly not widely known in the photovoltaic community) means that we can expect an increased effort from the materials community dedicated to the investigation of these materials as well as finding new ones.

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1. PV outputs are often given as peak watts (W_p) to reflect the fact that electricity is only generated when the Sun is shining, which averages out globally at around 5 hours a day. The subscript p is omitted in the rest of this article for simplicity. W refers to peak watt in all cases.
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NEUROSCIENCE

Path to Treat Rett Syndrome

Alan K. Percy

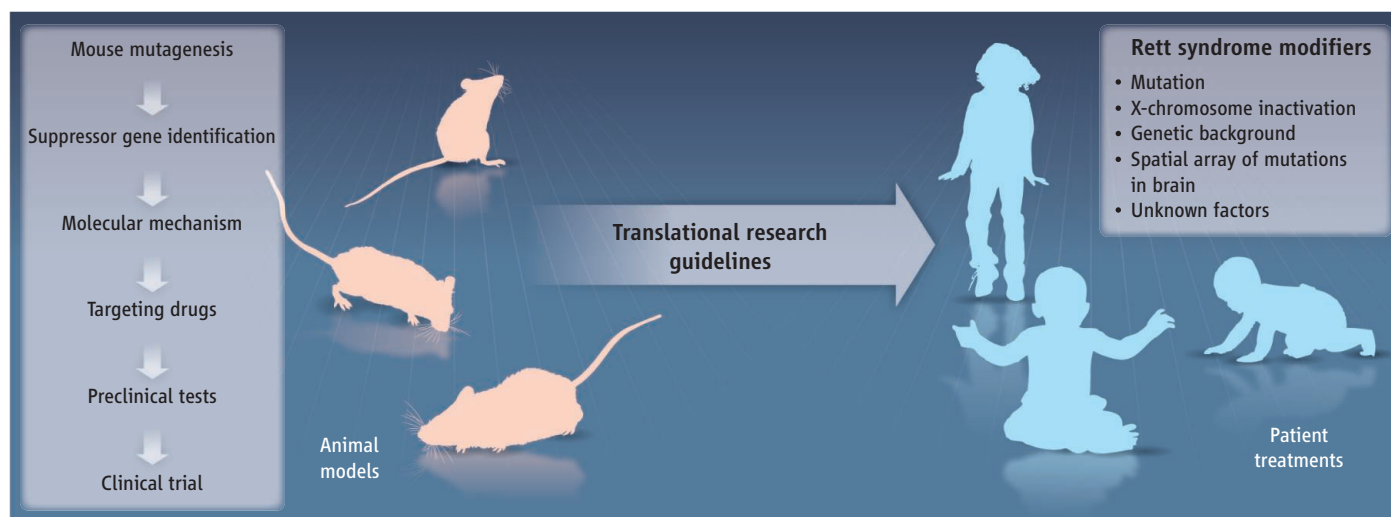
Rett syndrome (RTT), a unique neurodevelopmental disorder seen almost exclusively in females, was first recognized about 50 years ago by Andreas Rett (1), a developmental pediatrician in Vienna, and virtually simultaneously by Bengt Hagberg, a Swedish neurologist. However, not until a chance meeting of the two in 1981 were these observations crystallized, yielding the first widely read English-language description of RTT (2). Thereafter, RTT received considerable attention throughout the world. Concerted effort led to the identification of mutations in the gene *Methyl-CpG-binding protein 2 (MECP2)* in 1999 (3), where-

upon laboratory-oriented research exploded, including the development of numerous animal models. A key observation from an early mouse model indicated the possibility of substantial reversibility of the disease phenotype (4). Since then, translational research has been conducted in available mouse models to identify potential therapies including insulin-like growth factor 1 (IGF-1) (5), disabling GABAergic neuron function (6), and stem cell transplantation (7). Recently, intriguing results showed that alterations in cholesterol metabolism in response to statin therapy (8) had a beneficial effect on RTT-associated symptoms in a mouse model of the disease. This has prompted interest in advancing this treatment to humans.

Rigorous testing of potential disease-modifying factors of Rett syndrome is needed to guide research findings toward clinical trials.

The onset of RTT occurs most commonly between the ages of 6 months and 30 months, first with a plateau in development and then regression principally of fine motor and communication skills (9), accompanied by hypotonia, stereotypic movements, epilepsy (10), and autonomic difficulties (11). Abnormal head growth deceleration, markedly altered height and weight (12), and scoliosis (13) occur in most patients; many display transient autistic features. Considerable data have been generated through the Rett Syndrome Natural History Study (ClinicalTrials.gov identifier NCT00299312) to address the disorder over time and to generate phenotype-genotype associations. At present, no disease-modifying therapy is available. However, current clinical

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Guidance required. Moving from mutagenesis-based screens to clinical trials requires guidelines for high-quality animal model translational research.

There are several disease-modifying factors for Rett syndrome that determine clinical severity.

trials related to IGF-1 (NCT01777542 with recombinant human IGF-1; NCT01703533 with a tripeptide from IGF-1) are ongoing, and more are anticipated as translational research matures. The statin study suggests another potential disease-modifying agent (8).

Buchovecky *et al.* (8) made use of an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis suppressor screen in a *Mecp2*-null mouse model of RTT. This approach identified five suppressors that mitigated the effects of the null mutation in male mice. One of these suppressors involved an inactivating mutation in the *Sqle* gene, which encodes squalene epoxidase, the rate-limiting enzyme of cholesterol biosynthesis. Surprisingly, this suppression revealed an underlying defect in cholesterol amounts in the brain and liver of these animals. Statin drugs (fluvastatin and lovastatin) used in male *Mecp2*-null mice improved the concentration of cholesterol in these organs, reduced motor dysfunction, and improved longevity. Female mice harboring genetic perturbations in *Mecp2* that are seen in RTT patients also responded positively to fluvastatin (such as improved motor performance on the balance beam). These results suggest the importance of using such genetic screens. Further efforts to identify the basis for the remaining four identified suppressors in this model are awaited.

The intriguing finding regarding cholesterol metabolism and the response to statins was conducted in a mouse strain associated with obesity. Is this a finding, therefore, specific to this strain, or is it generalizable? Would a similarly robust effect be noted in “skinny” animals? Guidelines for high-quality animal model translational research (14)

were not followed in this exploratory study (8). The use of additional mouse strains as well as replication in another laboratory will be required before this treatment can advance to formal clinical trial.

In 2011, the U.S. National Institute of Neurological Disorders and Stroke and the Eunice Kennedy Shriver National Institute of Child Health and Human Development convened a workshop to discuss the relationship between RTT and available mouse models involving either deletion of *Mecp2* or the expression of disease-causing mutant forms of *Mecp2*, and to define standards for preclinical studies conducted with highest rigor (14). At about the same time, another study highlighted differences among various mouse models of RTT (15). Important differences exist between the onset of RTT in humans and in animal models. Male mice are typically symptomatic within the first 4 to 6 weeks of life; female animals are generally not symptomatic before several months of life. Both situations occur markedly later in the mouse life span relative to the human life span. In addition, different mouse strains may show differences in clinical abnormalities relative to those seen in humans. Moreover, certain mouse strains are obese relative to others. As such, guidelines for preclinical trials established the following parameters (14): study design (including sample size of at least 12 to 16 animals per group); statistical design (including blinding of investigators to treatment or mutation status) reporting both positive and negative findings; and replication (including the use of more than one model and independent laboratory replication).

The assessment of treatment efficacy in RTT is an important concern. Because RTT is an X chromosome-linked dominant disorder, the determination of clinical severity rests on several points (see the figure). With more than 250 different mutations identified, phenotype-genotype variation is considerable. As a single factor occurring alone, this should be a relatively straightforward consideration. However, two females with the same mutation may be markedly different in their clinical severity. Among the factors influencing this, X-chromosome inactivation (XCI; one of the two copies of the X chromosome in female mammals is inactivated), the spatial distribution of mutations within the brain, and the genetic background of the participant must be considered, as well as elements yet unknown. XCI in the blood of RTT patients may be heavily skewed in either direction—suggesting more or less severe clinical involvement for a specific mutation—but this issue has not been addressed in brain, the relevant tissue. The spatial distribution of mutations cannot be measured at present, and genetic background studies are only in formative stages. Although clinical severity can be measured, the many variables related to it in RTT remain to be fully understood. In addition, quality-of-life data derived from the Rett Syndrome Natural History Study revealed an inverse relation between motor and behavioral factors. Individuals with greater motor capabilities had more difficult behavioral issues, including oppositional and self-injurious behaviors (16). Consideration must be given to adopting treatment strategies that improve motor capabilities but might worsen behavior.

Nonetheless, the evidence of reversal the RTT mouse model of Buchovecky *et al.* represents an important finding regarding the possible implementation of a therapeutic trial if the published guidelines are satisfied. The importance of optimal guidance is absolutely essential in moving these efforts to effective clinical trials. Buchovecky *et al.* have opened up a surprising and exciting new avenue for drug development. Perhaps even more provocative is the identification of the four other genes as potential modifiers of disease in equally unexpected ways.

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MATERIALS SCIENCE

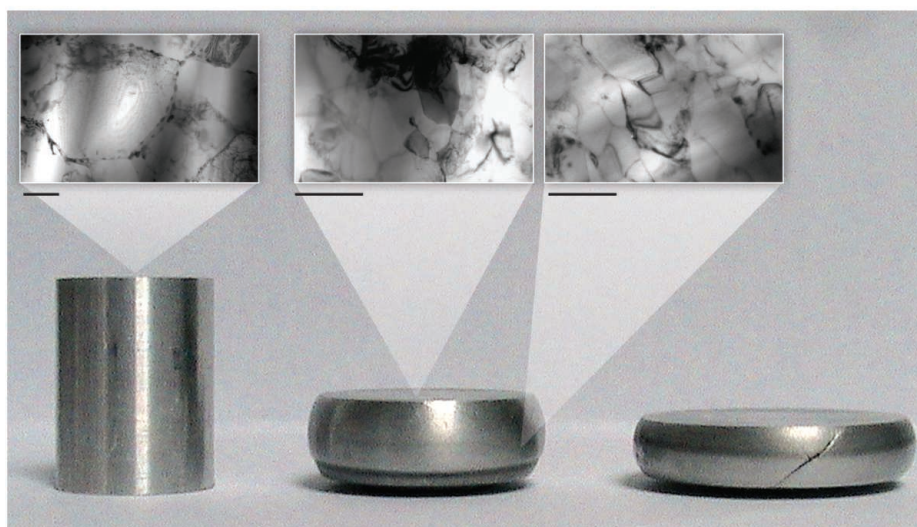
How to Be Both Strong and Thermally Stable

Salah Ramtani

Ultrafine-grained (UFG) materials with grain sizes below 1 μm have been a subject of extensive investigation over the past two decades because of their unusual properties, including resistance to wear, fracture strength, and resistance to corrosion even at high temperatures. On page 337 of this issue, Liu *et al.* (1) report a UFG material with a nanolaminated structure. The hardness and thermal stability of this material exceeds that of other known UFG materials. To produce the material, the authors apply a very high-rate shear deformation with high strain gradients to the top surface layer of a pure bulk nickel rod, thereby inducing the formation of two-dimensional nanometer-thick laminated structures. The resulting material has gradients in both geometrical grain structure and mechanical properties and also shows high thermal stability.

Nanograined and UFG metals can be 10 times as hard and strong as their conventional grain size counterparts, which have grain sizes of 10 μm or more. UFG materials have high yield strength (that is, they deform elastically up to high stress values), because grain boundaries typically impede dislocation movement. By changing grain size, the grain-boundary density also changes, which in turn influences dislocation movement and yield strength, an effect described by the Hall-Petch relationship (2–5).

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Strong and hard. An aluminum powder was first subjected to isostatic pressing to create a bulk consolidated material (left). This material was then impacted at high velocities, resulting in ultrafine-grained (UFG) materials (center and right) that were substantially harder than the original bulk material. Insets show the grain structure of the materials (6). In contrast to the materials shown here, the UFG material reported by Liu *et al.* has a nanolaminated structure that makes it particularly strong and stable.

To illustrate Hall-Petch strengthening, consider an UFG aluminum sample made from commercial-purity powder (see the figure) (6). The powder was first subjected to hot isostatic pressing, resulting in a bulk consolidated material with a random texture and a homogeneous microstructure of equiaxed grains with an average diameter of $\sim 2 \mu\text{m}$. The material was then impacted, using a falling weight with impact velocity of either 7.8 or 9.2 m/s. The resulting material has an aver-

age grain diameter of $\sim 500 \text{ nm}$, with a strong gradient of fiberlike crystallographic texture perpendicular to the impact direction (6).

However, UFG materials suffer from a lack of ductility, which is the ability to plastically deform without breaking. Low ductility is a common problem in high-strength materials such as ceramics and UFG materials. Artifacts from processing, force instability in tension, and crack nucleation from flaws limit the ductility of UFG materials (7).

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UFG materials can have some inherent ductility, but they tend to suffer from plastic instabilities upon deformation, limiting their use in structural applications. One example is the concentration of large deformation in shear bands. In tension, the necking instability sets in early (necking is a mechanism of tensile deformation where relatively large amounts of strain localize disproportionately in a small region of the material). Inhomogeneous deformation can lead to the onset of failure, severely limiting the useful ductility in UFG steels and other UFG metals and alloys (8). This seriously limits the use of these materials in structural applications.

The low ductility in UFG metals and alloys is mainly due to their very poor work-hardening capacity, caused by their inability to accumulate dislocations because of the smallness of the grain size and the initially high density of entangled, immobile dislocations in the case of samples processed by severe plastic deformation (9). The properties of nanograined and UFG metals have been a

major focus for computational materials science in recent years, particularly the behavior of the grain boundaries often associated with the atomic-level mechanisms of plastic deformation, as analyzed through experiments, atomistic simulations, theoretical models, and numerical investigations (10–12).

The material presented by Liu *et al.* is unusual in that it is both geometrically and mechanically graded and has exceptional properties. Its hardness exceeds that reported for UFG Ni, and its coarsening temperature is 40 K above that for UFG Ni. This study will open new perspectives for fundamental research and potential technological applications in a wide range of industrial manufacturing processes.

Although it remains challenging to produce the desired microstructures for structural applications, technological advances and computational tools make it increasingly more feasible to control and engineer the grain structure of materials to very fine details. The prospect of bimodal or multimodal grain

structures in improving the mechanical properties of UFG metals appears very promising (12, 13) for structural applications.

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PALEONTOLOGY

Did the Denisovans Cross Wallace's Line?

A. Cooper¹ and C. B. Stringer²

The recent discovery of Denisovans (1, 2) and genetic evidence of their hybridization with modern human populations now found in Island Southeast Asia, Australia, and the Pacific (3) are intriguing and unexpected. The reference specimen for the Denisovan genome (4), a distal phalanx from a young girl, was recovered from the geographically distant Denisova Cave in the Russian Altai mountains. Three Denisovan mitochondrial genomes have been generated from material in the cave, dated by poorly associated fauna (5) at more than 50,000 years old. The diversity of these genomes indicates that the Denisovan population had a larger long-term average size than that of the Neandertals (6, 7), suggesting that the Denisovans were formerly widespread across mainland East Asia. However, interbreeding with modern humans only appears to have

occurred in remote Island Southeast Asia, requiring marine crossings and raising questions about the distribution and fossil record of Denisovans in Island Southeast Asia.

The distribution of modern human populations containing detectable amounts of introgressed Denisovan DNA is surprising, as none have been detected in mainland Asia (introgressed DNA refers to small amounts of DNA from one species found in another species). Denisovan DNA has only been found on islands east of Wallace's Line (see the figure). The modern human populations with the highest percentage of Denisovan DNA are the geographically isolated New Guinean and Australian aborigines (~3 to 4%) (4), whereas smaller percentages have been detected in a range of populations in Island Southeast Asia. Groups in this area are thought to be descended from early Southeast Asian hunter-gatherers and later Neolithic farmers (3).

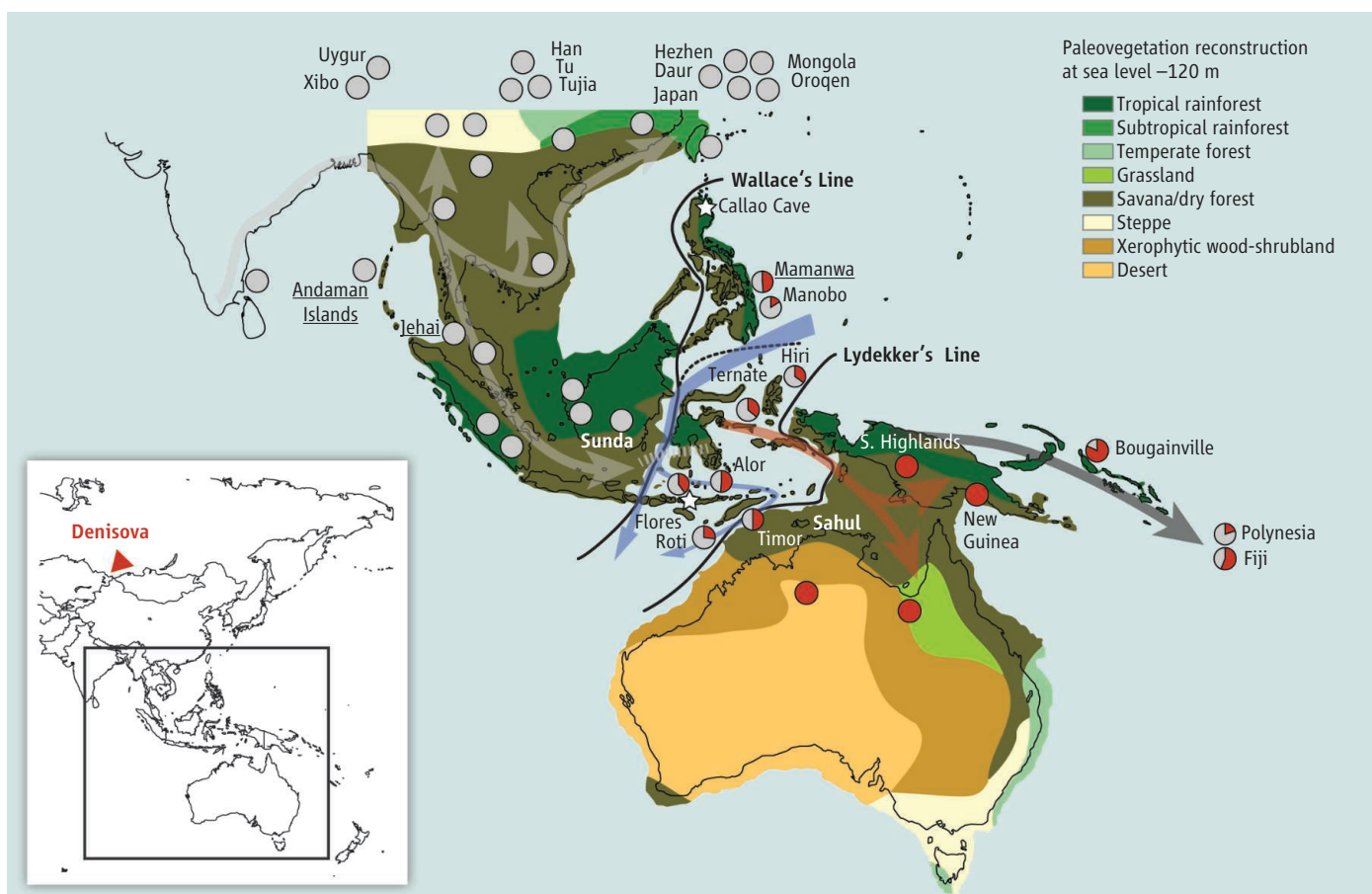
Wallace's Line (8) is one of the world's biggest biogeographic disjunctions, marking the border of placental-dominated eco-

The distribution of Denisovan DNA in modern human populations raises questions about where these ancient humans lived and where they interbred with modern humans.

systems to the west, whereas the lesser known Lydekker's Line marks marsupial-dominated ecosystems to the east (see the figure). Only two terrestrial mammal groups are known to have crossed Wallacea (the area between the two lines) to migrate into Australasia: rodents and anatomically modern humans. The discovery of *Homo floresiensis* ("Hobbits") on Flores in 2003 (9) indicates a separate dispersal across Wallace's Line, whereas a ~67,000-year-old foot bone from Callao in the Philippines represents a small-bodied hominin of unknown taxonomic affiliation (10). These taxa remain enigmatic, but suggest that other hominin species had the capacity to cross the powerful marine current that forms and maintains Wallace's Line even during times of lowered sea levels.

Denisovan populations appear to have had a diverse ecological range covering both mainland and Island Southeast Asia (5). The inferred large historical population size is consistent with the use of the extensive savannah regions on the exposed Sunda shelf as a refugium during Pleistocene glacial phases

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Ancient migrations. The proportions of Denisovan DNA in modern human populations are shown as red in pie charts, relative to New Guinea and Australian Aborigines (3). Wallace's Line (8) is formed by the powerful Indonesian flow-through current (blue arrows) and marks the limit of the Sunda shelf and Eurasian placental mammals. Lydekker's Line does the same for the Sahul shelf and marsupial mammals, with the area between the two lines known as Wallacea. An inferred coastal route of modern human colonization (light gray arrows) through paleovegetation zones (17) is shown on a -120 m paleoshoreline (18). Inter-

breeding with indigenous Denisovan populations is suggested to have occurred after the initial dispersal of modern humans across Wallace's Line (red arrows), with the genetic signals subsequently diluted to varying degrees and carried throughout the region and Pacific (dark gray arrows) by later Austronesian populations (3). In contrast, modern and indigenous hunter-gatherer populations (underlined) show no or very little Denisovan DNA west of Wallace's Line, suggesting limited back migration from Wallacea. Northern mainland populations are not shown in accurate geographical positions.

(11). The exposed shelf would have allowed northward and southward migration during climatic cycles.

The location of the Denisovan reference specimens in the Altai mountains might suggest that Denisovan DNA gene flow into modern human populations occurred somewhere on the Asian mainland, before spreading throughout the Southeast Asian region. The apparent absence of Denisovan introgression in current mainland populations is most easily explained through overwriting by the DNA of incoming East Asian populations in areas other than Island Southeast Asia. However, analysis of indigenous negrito/hunter-gatherer populations on mainland Malaysia and the Andaman Islands revealed no Denisovan DNA introgression, even though the long-isolated Andaman Islanders show no admixture with other East Asian populations (3). Similarly, genomic analysis of an ancient modern

human in China (Tianyuan, ~40,000 years old) detected no Denisovan DNA (12), arguing against the existence of a prehistoric interbreeding signal that has been overwritten.

Together, these observations argue against an ancient introgression of Denisovan DNA on the Asian mainland (3). Instead, the source of the Denisovan gene flow appears to have been east of Wallace's Line, with the lack of Denisovan DNA in mainland populations explained by Wallace's Line limiting the reverse dispersal of introgressed populations. Subsequent movements of East Asian/Neolithic modern humans appear to have diluted the Denisovan-introgressed populations outside Australia and New Guinea, and also carried the signal further throughout the area and across the Pacific (3).

The only well-characterized hominin known to have crossed Wallace's Line before modern humans is *H. floresiensis*, whose

affinities remain enigmatic, although morphological analyses suggest derivation from an early *Homo erectus* ancestor or an even more primitive species (9). A stone tool record on Flores dated to more than 1 million years supports an early presence (9). However, despite its location beyond Wallace's Line, trying to identify *H. floresiensis* as a regional representative of the Denisovans is difficult to reconcile with the enlarged molars of the Denisovans, and the divergence date of Denisovans and modern human populations estimated with mitochondrial DNA at ~1 million years ago (1), or 170,000 to 700,000 years ago with genomic data (4).

The considerable difference between these divergence date estimates is interesting, and may relate to recent reports that the Denisovan genome contains large amounts of introgressed Neandertal genomic DNA (6, 7). This will affect estimates of both the phy-

logenetic relationships and genomic divergence dates between Neandertals, Denisovans, and modern humans. Alternatively, the older mitochondrial divergence date of ~1 million years may reflect the input of a more ancient Asian population, or that all the dates are overestimates resulting from the temporal dependency of molecular rates, and the erroneously low rate produced by the distant chimp-human external calibration (13).

We thus infer that *H. floresiensis* was an endemic species whose lineage originated at least 1 million years ago, restricted to a small region of Wallacea, whereas the Denisovans probably arrived during the mid-Pleistocene (after 600,000 years ago) and spread more widely in the region. The Denisovans east of the Wallace line may be represented by the Philippines Callao specimen, or have not yet been recognized. Other enigmatic hominin remains in Asia—from Narmada (India) and Dali, Jinniushan, Maba, and Xujiayao (China)—may represent the apparently once more extensive Denisovan population, or perhaps yet other species.

The Denisovan genome reportedly also contains a small contribution from another archaic population, whose source is currently unknown (6, 7). Did the Denisovans interbreed with a more ancient species, such as *H. erectus* or *H. antecessor*, or perhaps a late surviving *H. heidelbergensis* in Asia (14)? Given the uncertainties in the molecular dates, the genomic divergences may be compatible with a recent model suggesting that modern humans, Neandertals, and Den-

isovans are a trichotomy that originated from the widely dispersed Middle Pleistocene species *H. heidelbergensis*, perhaps around 400,000 years ago (14). The fragmentary and disparate nature of the East Asian fossil record provides only tantalizing glimpses of a diversity of hominin groups. Similarly, the apparently widespread distribution of early hominins across Wallacea, exemplified by the finds from Flores and the Philippines, raises the issue of whether they could even have extended to the Sahul shelf and regions like New Guinea and Australia (see the figure).

Why did gene flow between Denisovans and modern human populations occur primarily east of Wallace's Line and not on the Asian mainland? Given that intentional dispersal to Wallacea required the use of watercraft, the first modern human groups encountering the established Denisovan populations were likely to have been of very limited size. Either interbreeding may be more likely under these circumstances, or any interbreeding that does occur is more likely to be preserved as a signal in descendants. The genomic evidence suggests that gene flow from the Denisovans may have been largely male-mediated, providing some clues about the nature of the interactions (4). In addition, rapid dispersal by modern humans into tropical Wallacea is likely to have led to exposure to a wide range of new pathogens, such that disease resistance alleles obtained through hybridization with native populations may have been selectively advantageous (15). The first groups

of modern humans leaving Africa, which were also presumably of limited size, similarly appear to have interbred during initial encounters with established Neandertal populations in western Asia (16). An anticipated wealth of new genomic data are set to further illuminate the nature of these interactions between Neandertals, Denisovans and modern humans, as well as the extent and possible functionality of the DNA that was exchanged.

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MATERIALS SCIENCE

Soft Acoustic Metamaterials

Thomas Brunet¹, Jacques Leng², Olivier Mondain-Monval³

Resonance phenomena occur with all types of vibrations or waves and may play a part in spectacular events, such as the collapse of structures—for example, the fall of the Broughton suspension bridge near Manchester in 1831 (1). Indeed, the oscillations of a structure submitted to harmonic excitation reaches its maximum amplitude at the resonance frequency ω_0 of the system. At low driving frequencies ($\omega < \omega_0$), its response is in phase

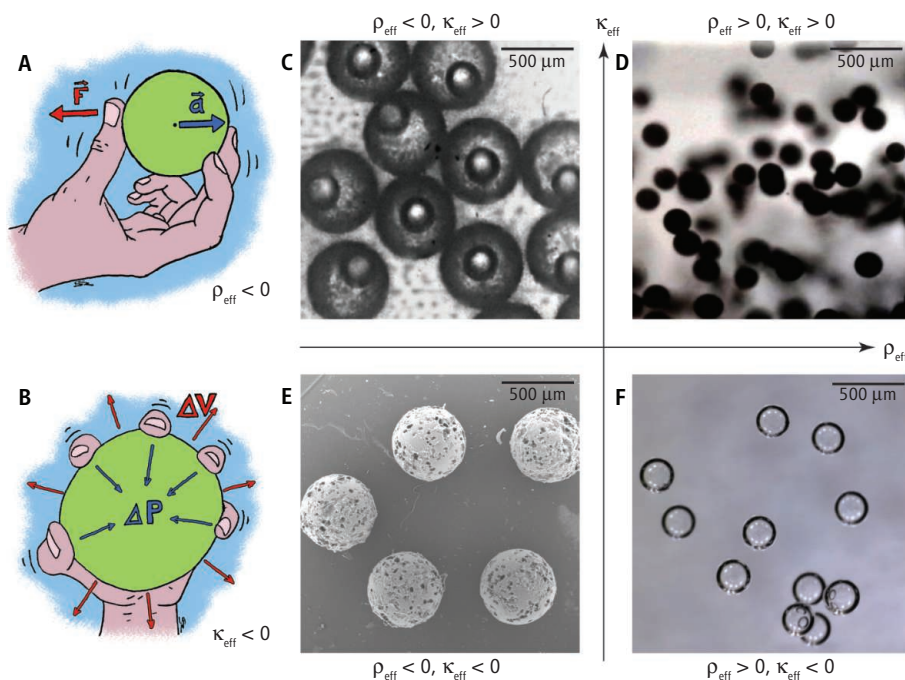
with the forcing but becomes out of phase just beyond ($\omega_0 < \omega$). Such an out-of-phase response has been exploited with “locally resonant materials” (2). The proposed strategy is to embed a large enough collection of identical mechanical resonators in a passive structure to control wave propagation. These features are used to reach unusual macroscopic behaviors such as ultradamping of noise or negative refraction for imaging (3).

The macroscopic frequency-dependent effective parameters (effective mass density ρ_{eff} and bulk modulus κ_{eff}) of such a composite can be easily derived if the resonators are much smaller than the incident acoustic wavelength. In the out-of-phase regime (ω_0

$< \omega$), ρ_{eff} and κ_{eff} may exhibit negative values. As illustrated in the figure, a negative mass density means that a volume element V_0 of the composite accelerates (vector a) in the opposite direction to the driving force F as $F = (\rho_{\text{eff}} V_0) a$ (see the figure, panel A). A negative bulk modulus implies that the composite expands upon an isotropic compression as $\Delta P = -\kappa_{\text{eff}} (\Delta V/V_0)$ (see the figure, panel B).

The effective refractive index is given by $n_{\text{eff}}^2 = \rho_{\text{eff}}/\kappa_{\text{eff}}$. When either ρ_{eff} or κ_{eff} is negative, depending on the nature of the resonators, n_{eff} becomes purely imaginary (evanescent waves), implying an exponentially decaying wave as sought for efficiently

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Features of acoustic metamaterials. (A and B) Schematic illustrations outline the dynamic behaviors of locally resonant materials with negative-valued effective parameters submitted to harmonic excitations. (A) The acceleration a of a material possessing a negative effective mass density ($\rho_{\text{eff}} < 0$) is opposite to the driving force F . (B) A material possessing a negative effective bulk modulus ($\kappa_{\text{eff}} < 0$) supports a volume expansion ($\Delta V > 0$) upon an isotropic compression ($\Delta P > 0$). (C to F) Classification of various locally resonant materials in the $(\rho_{\text{eff}}, \kappa_{\text{eff}})$ plane in terms of the sign of the effective mass density and the bulk modulus, made of various resonant inclusions: (C) core-shell particles, (D) “slow-oil” droplets, (E) polymer porous beads, and (F) air bubbles.

blocking low-frequency sound. For example, the dipolar resonance of core-shell particles induces a negative mass density (2), whereas the monopolar resonance of Helmholtz resonators implies a negative bulk modulus (4). Otherwise, negative refraction requires both ρ_{eff} and κ_{eff} to simultaneously exhibit negative values at the same frequency (5), which leads to a negative index (propagating waves) as sought for subwavelength imaging (6). Several resonant structures satisfying these two conditions have been proposed that benefit from simultaneous monopolar and dipolar resonances of two types of inclusions (7). Alternatively, another promising route is the exploitation of strong Mie-type resonances (the wavelength of the sound wave propagating within the scatterers being comparable to their size) of a single type of particles. The speed of sound within these particles should be very low in comparison with that of the host matrix in order to achieve a “double-negative” acoustic metamaterial (8).

Most of the reported experimental realizations in the audible domain have been for long-wave sound insulation by means of two-dimensional (2D) panels made of millimeter-scale, handmade resonators (9). For

other applications, such as subwavelength imaging, the achievement of a negative-refraction flat lens (10) calls for the manufacture of 3D resonant structures, which is far from obvious in the megahertz frequency range required for echography (e.g., ultrasonograms). Indeed, for ultrasound, the characteristic size of the resonators should be reduced to the micrometer scale, for which mechanical engineering becomes inadequate for a massive production of calibrated objects. Soft-matter methods, such as microfluidics, chemical formulation, or self-assembly, can be used to fabricate resonators with various shapes, structures, and compositions.

Such an approach requires the manipulated objects to be fabricated in soft phases like fluids, gels, or soft solids. Recently, microfluidics has allowed the production of core-shell particles with a submillimeter size (11) (see the figure, panel C). Otherwise, microfluidics is well adapted for an accurate control of both the size and the geometry of liquid inclusions, especially structured in a complex way (12). Recently, it allowed the production of highly monodisperse emulsions (see the figure, panel D) exhibiting a wide array of multipolar Mie resonances

(13) that result from the sound-speed contrast between fluorinated-oil droplets (~500 m/s) and a water-based host matrix (~1500 m/s). However, much higher sound-speed contrasts are required to enhance Mie-type resonances (3), and current efforts now focus on the synthesis of “ultraslow” inclusions, that is, dense yet soft particles.

Such requirements should be satisfied with a mesoporous material, in which the bulk modulus is strongly reduced (i.e., the material becomes softer) by the presence of a large amount of air cavities. This approach achieves a nonzero mass density because the mass of the solid skeleton, whereas the air bubbles contribute only to the negative bulk modulus (see the figure, panel F). Possibilities are offered by silica aerogels—well known for possessing a very low speed of sound (<100 m/s) (14)—or porous polymer materials, because they can be shaped as spherical beads (see the figure, panel E) using microfluidics (15).

Such “soft” approaches also offer the possibility to mold the fluid metamaterials in any possible shape thanks to its liquid or gel-like state. Another advantage is the ability of these fluid resonant structures to be highly responsive to any external fields (e.g., magnetic, electric, and shear) that can tune the material properties. This approach paves the way toward the realization of responsive and tunable acoustic metamaterials.

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Hyperdominance in the Amazonian Tree Flora

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Introduction: Recent decades have seen a major international effort to inventory tree communities in the Amazon Basin and Guiana Shield (Amazonia), but the vast extent and record diversity of these forests have hampered an understanding of basinwide patterns. To overcome this obstacle, we compiled and standardized species-level data on more than half a million trees in 1170 plots sampling all major lowland forest types to explore patterns of commonness, rarity, and richness.

Methods: The ~6-million-km² Amazonian lowlands were divided into 1° cells, and mean tree density was estimated for each cell by using a loess regression model that included no environmental data but had its basis exclusively in the geographic location of tree plots. A similar model, allied with a bootstrapping exercise to quantify sampling error, was used to generate estimated Amazon-wide abundances of the 4962 valid species in the data set. We estimated the total number of tree species in the Amazon by fitting the mean rank-abundance data to Fisher's log-series distribution.

Results: Our analyses suggest that lowland Amazonia harbors 3.9×10^{11} trees and ~16,000 tree species. We found 227 "hyperdominant" species (1.4% of the total) to be so common that together they account for half of all trees in Amazonia, whereas the rarest 11,000 species account for just 0.12% of trees. Most hyperdominants are habitat specialists that have large geographic ranges but are only dominant in one or two regions of the basin, and a median of 41% of trees in individual plots belong to hyperdominants. A disproportionate number of hyperdominants are palms, Myristicaceae, and Lecythidaceae.

Discussion: The finding that Amazonia is dominated by just 227 tree species implies that most biogeochemical cycling in the world's largest tropical forest is performed by a tiny sliver of its diversity. The causes underlying hyperdominance in these species remain unknown. Both competitive superiority and widespread pre-1492 cultivation by humans are compelling hypotheses that deserve testing. Although the data suggest that spatial models can effectively forecast tree community composition and structure of unstudied sites in Amazonia, incorporating environmental data may yield substantial improvements. An appreciation of how thoroughly common species dominate the basin has the potential to simplify research in Amazonian biogeochemistry, ecology, and vegetation mapping. Such advances are urgently needed in light of the >10,000 rare, poorly known, and potentially threatened tree species in the Amazon.

FIGURES AND TABLES IN THE FULL ARTICLE

Fig. 1. A map of Amazonia showing the location of the 1430 ATDN plots that contributed data to this paper.

Fig. 2. A rank-abundance diagram of 4962 tree species extrapolated to estimate the size of the Amazon tree flora.

Fig. 3. Characteristics of hyperdominant tree species of the Amazon.

Fig. 4. Proportions of hyperdominance by region and forest type.

Fig. 5. Distribution maps of three hyperdominant Amazon tree species.

Table 1. Population characteristics of the 20 most abundant tree species of the Amazon.

Table 2. Hyperdominance by region and forest type.

SUPPLEMENTARY MATERIALS

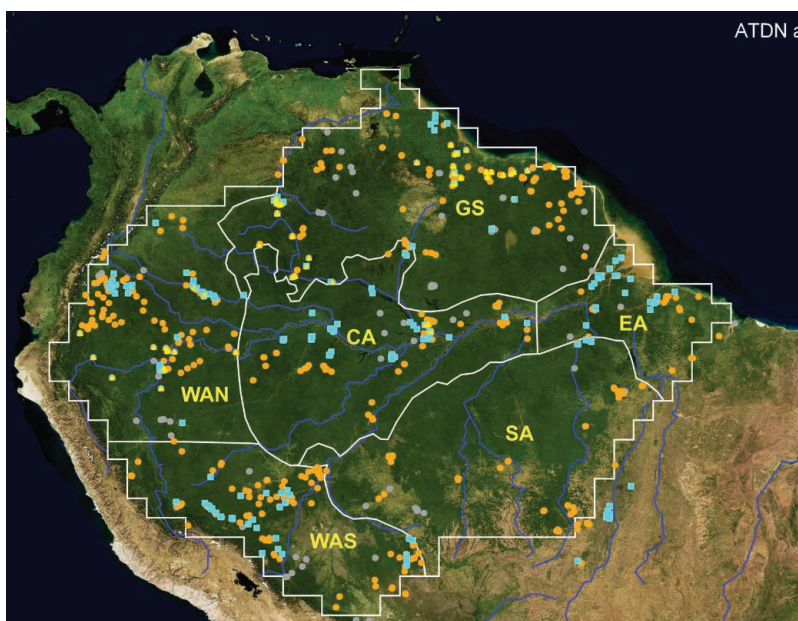
Supplementary Text

Figs. S1 to S12

Tables S1 to S3

Appendices S1 to S4

References (53–67)



A map of Amazonia showing the location of the 1430 Amazon Tree Diversity Network (ATDN) plots that contributed data to this paper. The white polygon marks our delimitation of the study area and consists of 567 1° grid cells (area = 6.29 million km²). Orange circles indicate plots on terra firme; blue squares, plots on seasonally or permanently flooded terrain (várzea, igapó, swamps); yellow triangles, plots on white-sand podzols; gray circles, plots only used for tree density calculations. Background is from Visible Earth. CA, central Amazonia; EA, eastern Amazonia; GS, Guyana Shield; SA, southern Amazonia; WAN, northern part of western Amazonia; WAS, southern part of western Amazonia. More details are shown in figs. S1 to S3.

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Hyperdominance in the Amazonian Tree Flora

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The vast extent of the Amazon Basin has historically restricted the study of its tree communities to the local and regional scales. Here, we provide empirical data on the commonness, rarity, and richness of lowland tree species across the entire Amazon Basin and Guiana Shield (Amazonia), collected in 1170 tree plots in all major forest types. Extrapolations suggest that Amazonia harbors roughly 16,000 tree species, of which just 227 (1.4%) account for half of all trees. Most of these are habitat specialists and only dominant in one or two regions of the basin. We discuss some implications of the finding that a small group of species—less diverse than the North American tree flora—accounts for half of the world's most diverse tree community.

Much remains unknown about the Amazonian flora, the richest assemblage of plant species on Earth. Tree inventories carried out over the past two decades have helped improve our understanding of regional-scale patterns of distribution and abundance in Amazonian tree communities, but similar advances at the basin-wide scale remain scarce. Scientists still do not know how many tree species occur in the Amazon (*1*), how many tree species have been recorded to date in the Amazon, how those species are distributed across the basin, and in what regions or forest types they are rare or common. So uncertain are patterns at the largest scales that even the simplest question of all—what is the most common tree species in the Amazon?—has never been addressed in the scientific literature, much less answered.

In practical terms, this lack of basic information means that the largest pool of tropical carbon on Earth remains a black box for ecologists. Also, given that abundance and frequency are the primary currencies of conservation status, there is essentially no information on which Amazonian tree species face the most severe threats of extinction and where to protect them. Although ecologists have an ever-larger toolbox of methods to extrapolate from local surveys to larger-scale patterns, well-known barriers to sampling, identifying, and putting valid names on tropical trees have long been assumed to make Amazon-wide extrapolations impossible.

We challenged those assumptions with a wide-ranging assessment of the composition and biogeography of Amazonian tree communities (*2*).

We compiled stem density and species abundance data from 1170 tree inventory plots across the Amazon (Fig. 1), well distributed among all regions and major forest types (table S1 and figs. S1 to S3), to generate basin-wide estimates of the abundance, frequency, and spatial distribution of thousands of Amazonian tree species.

Results

A Rank-Abundance Distribution for Amazonian Trees

The plots contained a total of 4962 valid species, 810 genera, and 131 families of trees [free-standing stems ≥ 10 cm in diameter at breast height (dbh)]. By using stem density and species abundance data collected in the individual plots, we constructed a spatial model that yielded estimated basin-wide population sizes for every valid species in the data set. The rank-abundance distribution (RAD) of these data (Fig. 2) offers four important insights regarding Amazonian tree communities.

First, it provides the most precise estimates yet of two numbers that have been debated for decades: How many trees and how many tree species occur in the ~ 6 -million- km^2 landscape of Amazonia (*1, 3–5*). Our estimate of tree density yielded a total of 3.9×10^{11} individual trees and a median tree density of 565 trees/ha (fig. S4). Assuming that our population size estimates for the common species are reasonable (fig. S5) and that Fisher's log-series model fits our data (table S2 and figs. S6 and S7) (*1*), we estimated the total number of tree species in the Amazon to be about 16,000 (Fig. 2). A second estimate based on the Fisher's alpha scores of all plots yielded a similar figure: 15,182 species (fig. S8).

Second, the RAD suggests that just 227 (1.4%) of the estimated 16,000 species account for half of all individual trees in Amazonia. We refer to these species, all of which have estimated populations of $>3.7 \times 10^8$ trees, as hyperdominant species (see a list of the 20 most abundant species in Table 1 and a full list in appendix S1). These hyperdominant species form the basis of the tree communities in individual plots as well, accounting for a median of 41% of trees (range = 0 to 94%, fig. S9) and 32% of species (range = 0 to 78%) per plot (fig. S9).

Third, all species ranking in abundance from 5000 to 16,000 are very rare. These species in the tail of the RAD have total populations of $<10^6$ individuals and together account for just 0.12% of all trees in Amazonia. Although some of these species may be treelets or climbers rarely reaching tree stature or “vagrants” spilling over from extra-Amazonian biomes such as the Cerrado and Andes, thousands must be Amazonian endemics that run a high risk of going extinct, even before they can be found and described by biologists. The rarest 5800 species have estimated population sizes of <1000 , which is sufficient to classify those that are endemic as globally threatened (*6*). Together, these taxa (the rarest 36% of species)

account for just 0.0003% of all trees in Amazonia. Given the extreme unlikelihood of locating a fertile individual of one of these species, we believe that discovering and describing the unknown portion of Amazonian biodiversity will be a long-term struggle with steeply diminishing returns and not an easy linear process (7). Indeed, the RAD suggests that floras of even well-collected areas may remain half-finished for decades. For example, our model predicts that ~4500 tree species occur in the Guianas (fig. S10), but centuries of collecting there have yielded just half that number (8). Some of these species may be present among the unidentified species of our plots or as undescribed specimens in herbaria (9), but the majority may yet have to be collected.

Fourth, there are strong similarities between theoretical models of tree species richness in the Amazon (1) and our distribution of species abundances based on empirical data. For example, Hubbell *et al.* (1) used a log-series distribution to predict that the most common species in the Amazon should account for 1.39% of all trees. This is very close to our estimate for the most common species in our data set, the palm *Euterpe precatoria* (1.32%). Our empirical estimate of

Fisher's alpha for the Amazon (fig. S8) is also extremely close to Hubbell *et al.*'s modeled prediction [754 versus 743 in (1)]. Although these strong correlations between predictions and our data set suggest that the log series may offer useful insights on the most poorly known tree species in the Amazon (e.g., the number of undescribed taxa), they should not necessarily be interpreted as evidence for any one theory of how these tree communities are structured (10, 11).

Hyperdominant Patterns in Regions, Forest Types, and Taxonomic Groups

We examined species' geographic ranges and abundances by plots, regions, and forest types to explore how hyperdominant species differ from other taxa, as a first step toward understanding what makes them so successful. Hyperdominant species have larger ranges than other taxa (Fig. 3A) and reach greater maximum relative abundances in plots (Fig. 3B). Most hyperdominant species (121 out of 227) are habitat specialists (Fig. 3C) [i.e., they show a strong preference for one of the five major Amazonian forest types: terra firme (53 spp.), várzea (26), white-sand forest (16), swamps (14), and igapó (12)]. Likewise, most are only

dominant within one or two forest types. When the study area was divided into six regions (Guiana Shield and northwest, southwest, south, east, and central Amazonia), most hyperdominant species (73%) were found to be dominant within only one or two regions (Table 2).

It is thus important to emphasize that, although the Amazonian RAD is dominated by a small suite of species, most of those species are only dominant in certain forest types and in certain regions of the basin. Just one species qualified as dominant in all six regions (*Eschweilera coriacea*), no species were dominant in all five forest types, and only four species were dominant in four forest types (*E. precatoria*, *Oenocarpus bataua*, *Licania apetala*, and *Euterpe oleracea*). Much more representative of the 227 hyperdominant species are taxa like *Siparuna decipiens* (112th largest population size overall), only dominant in terra firme forests in southwest Amazonia, and *Eperua falcata* (13th), only dominant in the Guiana Shield. Indeed, 58% of hyperdominant species qualify as both dominant in one or two regions and dominant in one or two forest types.

Within each region, an even smaller number of species (75 to 163) typically accounts for

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50% of all individual trees, and most of these regional dominants are also hyperdominant species (Fig. 4A). For example, the data suggest that half of all individual trees in southwest Amazonia belong to just 76 species, 50 of which are also hyperdominant species. The same pattern holds for forest types, which are individually dominated by 25 to 195 species (Fig. 4B). Half of all individual trees in white-sand forests belong to just 25 species, 15 of which are also hyperdominant species. Because most hyperdominant species are only dominant in one or two regions or forest types, in any single region or forest type the majority of the 227 hyperdominant species are not locally dominant.

Given these results, it seems likely that the basinwide patterns of dominance we describe here arise in part from regional-scale patterns of dominance described previously at various sites in upper Amazonia (12, 13). There is substantial compositional overlap between Pitman *et al.*'s (12) "oligarchies" in Peru and Ecuador and our hyperdominant species, even though those authors' plots represent just 2.1% of the full Ama-

zon Tree Diversity Network (ATDN) data set and only include terra firme forests. Sixty-eight "oligarchs" of (12) are on the list of 227 hyperdominant species, including 8 of the top 10 most common hyperdominants. The 250 oligarchic species in (12) account for 26.9% of all trees in Amazonia, according to the RAD in Fig. 2. These results suggest that the regional-scale and Amazon-wide patterns derive from similar processes.

Hyperdominants are more frequent in some families (table S3). Arecaceae, Myristicaceae, and Lecythidaceae have many (~four to five times) more hyperdominant species than expected by chance, whereas Myrtaceae, Melastomataceae, Lauraceae, Annonaceae, and Rubiaceae have fewer, probably because many of their species are shrubs or treelets that do not reach our 10-cm-diameter cutoff. In Fabaceae, the most abundant and most diverse family in the data set, the observed number of hyperdominant species is not significantly different from the expected.

We observed a negative relationship between the number of species in a genus and the frequency of hyperdominant species (fig. S11). This

pattern has been observed in several plant communities worldwide, and scientists have yet to determine whether it is ecologically informative or an artifact of rank-based taxonomy (14, 15). The 227 hyperdominant species belong to 121 genera, and 68 of these contain more hyperdominants than expected by chance (appendix S3). The highest number of hyperdominant species is found in moderately diverse *Eschweilera* (52 species overall; 2.4 hyperdominant species expected versus 14 observed), also the most abundant genus in the ATDN data set (5.2% of all stems). Given that the families and genera mentioned here dominate Amazonian forests, it remains a key goal to determine why some achieve dominance with a large number of mostly rare species (e.g., *Inga*, Sapotaceae) whereas others do so with a small number of common species (palms), differences that may result from variation in speciation and extinction rates (14–17). Although genetics data may reveal some hyperdominant species to be species complexes, there is not yet enough knowledge on how widespread such complexes are, where they are located along our RAD,

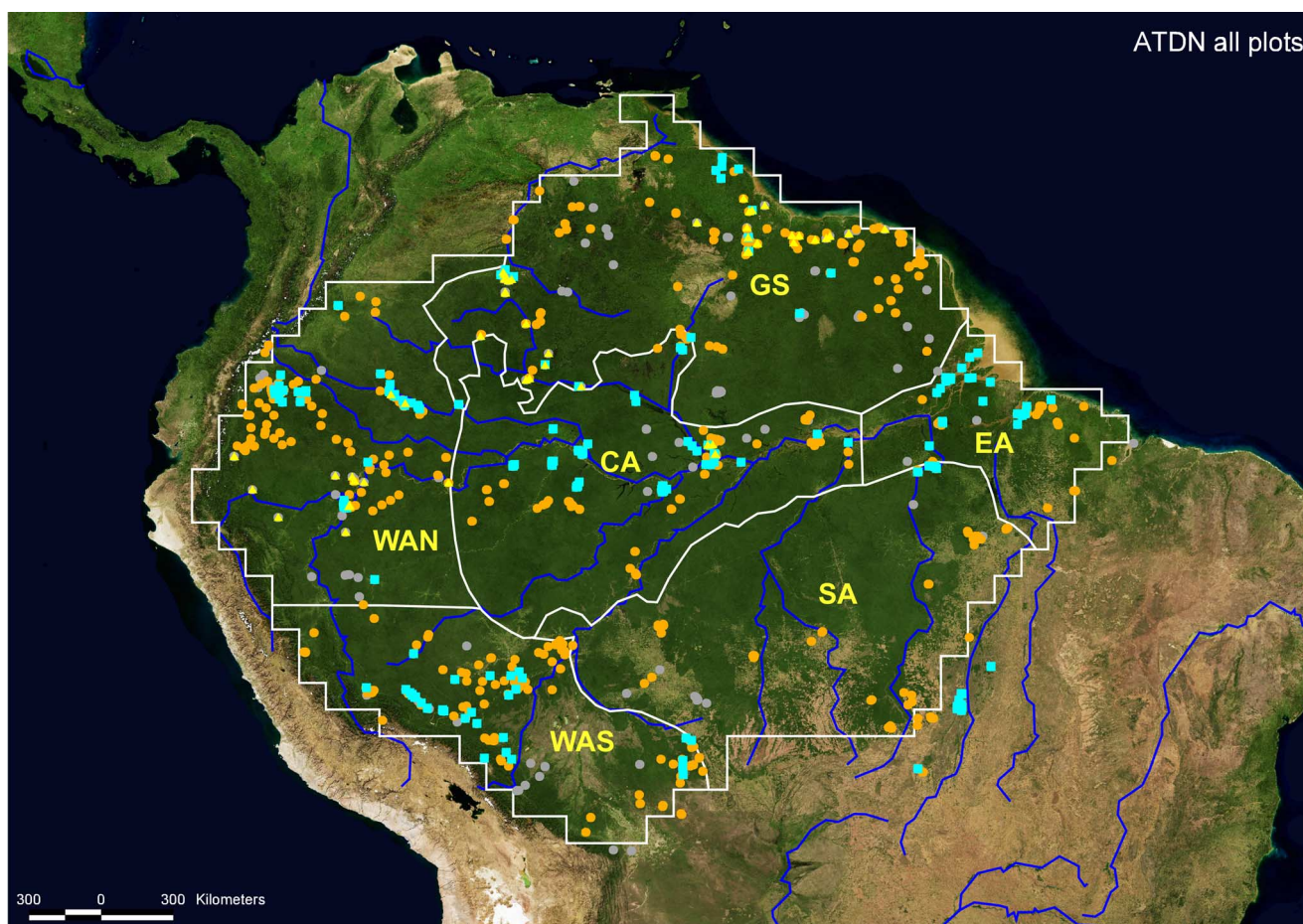


Fig. 1. A map of Amazonia showing the location of the 1430 ATDN plots that contributed data to this paper. The white polygon marks our delimitation of the study area [with subregions after (33)] and consists of 567 1°-grid cells (area = 6.29 million km²). Orange circles indicate plots on terra firme; blue squares, plots on seasonally or permanently flooded terrain

(várzea, igapó, and swamps); yellow triangles, plots on white-sand podzols; gray circles, plots only used for tree density calculations. Background is from Visible Earth (52). CA, central Amazonia; EA, eastern Amazonia; GS, Guyana Shield; SA, southern Amazonia; WAN, northern part of western Amazonia; WAS, southern part of western Amazonia. More details are shown in figs. S1 to S3.

and to what degree they could alter the patterns described here [(18) and references therein].

Discussion

Exploring Potential Causes for Hyperdominance

We found no evidence that two key functional traits for trees, seed mass and wood density,

vary consistently with hyperdominance. The 227 hyperdominant species include both shade-tolerant, typically large-seeded climax species with dense wood (e.g., *Chlorocardium rodiei*, *Clathrotropis* spp., and *Eperua* spp.) and shade-intolerant, small-seeded pioneers with light wood (e.g., *Cecropia* spp., *Jacaranda copaia*, and *Laetia*

procera). Given that most hyperdominant species attain very high local densities (>60 trees/ha) somewhere in the plot network, we predict that they will be found to be disproportionately resistant to pathogens, specialist herbivores, and other sources of frequency-dependent mortality (19, 20).

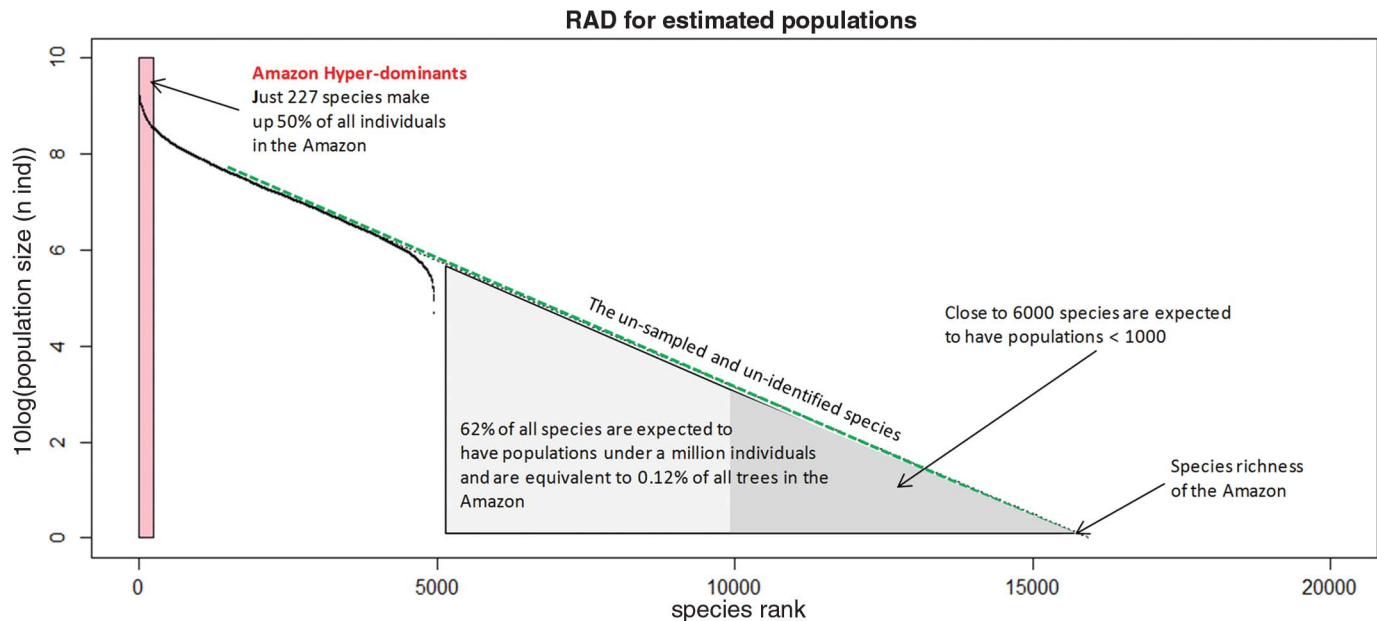


Fig. 2. A rank-abundance diagram of 4962 tree species extrapolated to estimate the size of the Amazon tree flora. The mean estimated Amazon-wide population sizes of 4962 tree species are shown as a solid line, and the dotted line is an extrapolation of the distribution used to estimate the total number of tree species in Amazonia.

Table 1. Population characteristics of the 20 most abundant tree species of the Amazon. Mean estimated population sizes of the 20 most abundant tree species in Amazonia and the empirical abundance and frequency data on

which the estimates were based. Median values for the 207 other hyperdominant species and for the 4735 other valid species in the data set are provided for comparison. Data on all species can be found in appendix S1.

Species	Mean estimated population in the Amazon	SD estimated population (%)	No. trees in data set	% of all plots where present	Maximum abundance recorded (trees/ha)
<i>Euterpe precatoria</i>	5.21×10^9	9.9	5903	32.7	168
<i>Protium altissimum</i>	5.21×10^9	18.0	5889	15.6	128
<i>Eschweilera coriacea</i>	5.00×10^9	5.6	9047	47.9	28
<i>Pseudolmedia laevis</i>	4.30×10^9	8.9	5285	36.1	121
<i>Iriartea deltoidea</i>	4.07×10^9	13.1	8405	18.5	169
<i>Euterpe oleracea</i>	3.78×10^9	17.5	8572	7.4	397
<i>Oenocarpus bataua</i>	3.71×10^9	10.7	4767	29.9	108
<i>Trattinnickia burserifolia</i>	2.78×10^9	29.4	3023	10	125
<i>Socratea exorrhiza</i>	2.68×10^9	10.8	863	28.6	82
<i>Astrocaryum murumuru</i>	2.41×10^9	11.2	5748	16.7	325
<i>Brosimum lactescens</i>	2.28×10^9	10.0	2234	28.2	106
<i>Protium heptaphyllum</i>	2.13×10^9	32.2	1365	11.3	169
<i>Eperua falcata</i>	1.95×10^9	15.8	1898	10.9	266
<i>Hevea brasiliensis</i>	1.91×10^9	15.5	6031	14.8	179
<i>Eperua leucantha</i>	1.84×10^9	32.3	1453	1.4	282
<i>Helicostylis tomentosa</i>	1.79×10^9	25.6	1948	36.5	89
<i>Attalea butyracea</i>	1.78×10^9	16.2	2561	5.8	73
<i>Rinorea guianensis</i>	1.69×10^9	18.6	1243	13.7	182
<i>Licania heteromorpha</i>	1.57×10^9	14.4	2483	35	173
<i>Metrodorea flavida</i>	1.55×10^9	14.7	1326	7.7	128
Median of other hyperdominant species	5.79×10^8		808	11.4	60
Median of non-hyperdominant species	1.11×10^7		15	0.5	5

Widespread pre-1492 cultivation by humans is a compelling hypothesis to explain hyperdominance (21). Numerous hyperdominant species are widely used by modern indigenous groups (*Hevea brasiliensis*, *Theobroma cacao*, and many palms), and some are associated with pre-Columbian settlements (*Attalea butyracea*, *A. phalerata*, *Mauritia flexuosa*) (22–26). On the other hand, most hyperdominant species are not commonly cultivated; many of the most commonly used hyperdominants (palms) belong to a family that appears to have been dominant in tropical South America since the Paleocene (27), and large portions of the Amazon Basin do not appear to have been heavily cultivated before 1492 (28).

Testing the Validity of the Model Predictions

A fundamental assumption of our analyses is that the population-size estimates generated by

the loess model were reasonably accurate for the most abundant species. This assumption is disputable for a few reasons: (i) The data set is very small compared with the community to which it was extrapolated; (ii) tree plots were not distributed randomly across the study area; (iii) trees were identified by many different research teams; and (iv) no environmental data were used by the model, even though many species in the ATDN data set are known to respond to environmental heterogeneity in the study area. A fifth problem makes the assumption especially difficult to test: (v) the fact that a basinwide population size has not been empirically determined for any Amazonian tree species, which precludes a comparison between projected and observed values. Here, we address these shortcomings by attempting to quantify the error that each could introduce into our results.

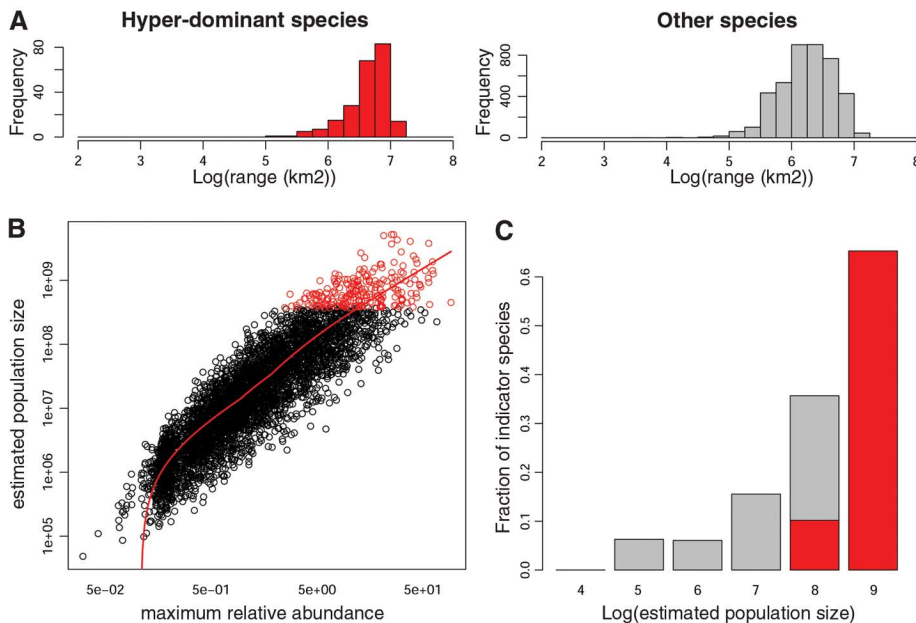


Fig. 3. Characteristics of hyperdominant tree species of the Amazon. (A) Hyperdominant species (red) have larger geographic ranges than other species (gray), (B) reach higher maximum relative abundances in individual plots, and (C) are more likely to be habitat specialists.

Table 2. Hyperdominance by region and forest type. The number of hyperdominant species that are also dominant in individual forest types and regions. Most hyperdominants only dominate a single forest type, and most are dominant in one or two regions.

		No. forest types where dominant						
		0	1	2	3	4	5	total
No. regions where dominant	0	3	3	0	0	0	0	6
	1	18	47	8	0	0	0	73
	2	12	65	12	3	0	0	92
	3	2	17	4	1	1	0	25
	4	0	9	3	5	0	0	17
	5	0	6	1	4	2	0	13
	6	0	1	0	0	0	0	1
total		35	148	28	13	3	0	227

To test how sampling intensity and the geographic distribution of plots (problems i and ii) affected the estimated population sizes of hyperdominant species, we recorded the frequency with which the 227 hyperdominants qualified as hyperdominant in the 500 runs of the bootstrap exercise described in the methods section. Most species (137, 60% of the total) qualified as hyperdominants in 90 to 100% of runs, whereas 207 species (91.2%) qualified as hyperdominants in more than half of runs (fig. S12A). Median (fig. S12B) and mean (fig. S12C) ranks for the 500 runs showed high stability.

In bootstrap runs for which a given hyperdominant species did not qualify among the top 227 species, it rarely qualified as rare. The lowest median rank observed for a hyperdominant species in the 500 bootstrap runs was 275, and hyperdominant species never ranked lower than 1000th (i.e., ranks 1000 to 4790). These analyses provide strong evidence that the identities and estimated population sizes of the hyperdominant species remain stable and predictable with varying levels of sampling intensity and geographic bias.

Taxonomic and identification problems (problem iii) are widespread in Amazonian tree inventories. However, two independent lines of evidence suggest that resolving these problems will not fundamentally alter the patterns described for hyperdominant species.

First, we observed a consistent relationship in the ATDN data set between the abundance of a species and the likelihood that it had been identified with a valid name. The percentage of identified species in individual plots was significantly higher than that of unidentified species-level taxa (87 versus 13% stems/ha, analysis of variance, $F_{5,22774} = 22,774$, $P < 0.001$). Furthermore, very common morphospecies are very infrequent in the ATDN data set. Only 48 of the 1170 ATDN plots contained a morphospecies that accounted for >10% of all individuals, and only 10 plots contained a morphospecies that reached >20%. Given that all 227 hyperdominants reach high local relative abundances (Fig. 3B), these numbers suggest that very few currently unidentified species will eventually qualify as hyperdominant species.

Second, we see strong evidence that taxonomic and identification problems are less severe in hyperdominant species than in other species, in the form of a strong positive correlation between the abundance of a species in the field, the number of specimens in herbaria, and the number of fertile specimens (i.e., specimens with flowers or fruits) collected during field work. Common species are better represented in herbaria than rare species, because individual collectors are more likely to encounter them (29). Common species are also more likely than rare species to be collected fertile during the establishment of tree plots. For example, in 25 ATDN plots established in eastern Ecuador (30), we found that hyperdominant species were more likely than other species to be collected fertile (27.8 versus 17.7%). Botanists trying to identify a hyperdominant species thus

have both a higher likelihood of matching their field specimens with museum specimens and a broader range of morphological features to facilitate identification.

The model we used to estimate population sizes was a loess function, parameterized exclusively with plot location and observed species abundances in plots and no environmental data (problem iv). This is a very different approach from the most commonly used class of species distribution modeling: maximum entropy modeling or Maxent (31, 32). Maxent uses presence-only data fitted to environmental variables of confirmed locations to produce a map of habitat suitability. In a Maxent model, a species known to occur under a given set of environmental conditions is predicted to occur in all environmentally similar areas, even when those areas are outside of the species' known range. Because Amazonian tree species are known to respond strongly to environmental variation, an earlier version of our model included climatic data. That version, however, routinely predicted significant populations of species in regions of the Amazon where a large number of ATDN plots and other plant collection efforts had consistently failed to record those species (i.e., type I errors were common). Modeling with only latitude and longitude as predictive variables is a more conservative option, because it ensures that such errors will be made at a much lower frequency and that species will never be predicted far from confirmed records (Fig. 5). For the same reason, we used a span of 0.2; at higher span values, species ranges extended too far into areas with no known occurrence. Varying span values from 0.2 to 0.5 did not strongly affect population size estimates.

It is not possible to compare estimated population sizes with measured population sizes (problem v), because the latter do not exist for any Amazonian tree species. However, it is possible to compare the population sizes estimated by the loess model with population sizes estimated by using a different method based on the measured extent of Amazonian forest types. The estimated population of *Mauritia flexuosa* is 1.5 billion stems. If we assume that one hectare of monodominant *M. flexuosa* swamp contains 565 *M. flexuosa* trees, then our 1.5-billion-stem estimate suggests that there are <3 million ha of monodominant *M. flexuosa* swamps in the entire basin. This appears reasonable, because the largest block of largely monodominant *M. flexuosa* stands in the basin (the Pastaza Fan) measures ~2.2 million ha. A similar test for white sands and podzol using *E. falcata* and *E. leucantha* (lumped together) was carried out. Together the model estimates that 3.9 billion trees in the greater Amazon belong to these species. If we assume that one hectare of white-sand [podzols and albic arenosols (33)] forest contains on average 150 stems that belong to these species, then the model suggests that there are roughly 26 million ha of white-sand and podzol forest in the greater Amazon. The extent of podzols in the greater Amazon has been estimated as 17 million ha (34). The estimate of podzols and arenosols (fig. S2) is 34 million ha (33).

We know of one study that attempted to estimate populations of trees over a large area in the Amazon Basin based on forest inventories of trees >30 cm dbh (35). The most abundant species in central western Amazonia (blocks: Roraima-Boa Vista, Manaus, and Rio Purus; total forest area 623,139 km²) was *E. coriacea*, with an esti-

mated population of 193 million individuals (this compares to roughly 800 million trees with >10 cm dbh), followed by *Goupia glabra* (93 million individuals, or 370 million trees with >10 cm dbh). Rollet concluded that *E. coriacea* is likely the most common tree species in the Brazilian Amazon. Although our data suggest that two other species have higher total population sizes (*E. precatória* and *Protium altissimum*), a difference caused by our much larger study area (~10×) and lower diameter cutoff (four times as many trees ha⁻¹), our estimate of *E. coriacea* (~5000 million) is of a similar order of magnitude (193 million × 10 × 4 = 7000 million). It is also worth noting that, in the forest inventories used by Rollet, other *Eschweilera* species were pooled more often with *E. coriacea* than in our inventories [see (36, 37) for a discussion on this].

Practical Implications

The finding that Amazonia is dominated by just 227 tree species has important practical implications. It suggests that roughly half of all fruits, flowers, pollen, leaves, and biomass in the world's most diverse forest belong to a very small suite of species, which must therefore account for a large proportion of Amazonian ecosystem services, including water, carbon and nutrient cycling. Our data also suggest that it may be possible to forecast a substantial proportion of the tree community composition and structure of unstudied sites in Amazonia with a purely spatial model. Although no one should underestimate the importance of the >10,000 rare and poorly known tree species in the Amazon (38), an appreciation of how thoroughly common species dominate the basin has the potential to greatly simplify

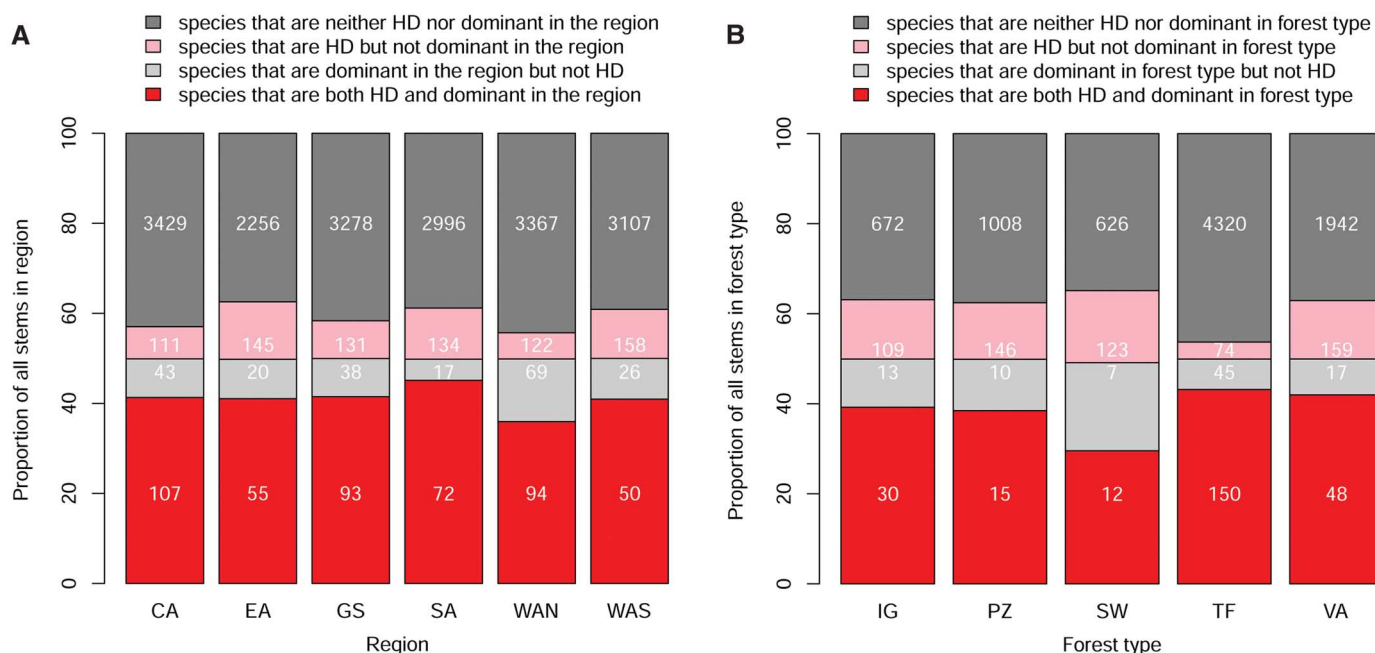


Fig. 4. Proportions of hyperdominance by region and forest type. (A) Proportions of the trees in each region belonging to species that are regionally dominant, hyperdominant, or neither. **(B)** Proportions of the trees in each forest

type belonging to species that are dominant in that forest type, hyperdominant, or neither. White integers show the number of species in each compartment. IG, igapó; PZ, podzol; SW, swamp; TF, terra firme; VA, várzea.

research in Amazonian biogeochemistry, plant and animal ecology, and vegetation mapping.

Materials and Methods

The ATDN network (39) comprises 1430 tree inventory plots distributed across the Amazon Basin and Guiana Shield, hereafter Amazonia (Fig. 1). Plots were established between 1934 and 2011 by hundreds of different botanists, some working in basinwide or global networks (39–42). Analyses of tree density were performed by using the 1346 plots with trees with ≥ 10 cm dbh that remained after plots with outlying density values (<100 or >1000 individuals/ha), poorly defined areas, or a different diameter cutoff level were removed.

Analyses of composition were performed with a subset of 1170 plots in which all 639,639 free-standing trees with ≥ 10 cm dbh had been iden-

tified with a valid name at the species (86.6%), genus (96.9%), or family (98.9%) level before our study. Most plots (852) measured 1 ha; 253 were smaller, 61 were larger, and 4 were plotless samples (point-centered quarter) for which the sampled area was unknown but the number of trees was equivalent to that typically found in 0.5 to 1 ha. We did not compare specimens or reidentify trees from these plots but resolved major nomenclatural issues (i.e., synonyms and misspellings) in the existing data sets by cross-checking all names with the TROPICOS database (43), via the Taxonomic Name Resolution Service [TNRS (44) (version October 2011)]. We made two adjustments to the names given in TROPICOS (supplementary text). *Rollinia* was merged with *Annona*, because phylogenetic analysis has revealed it to be nested inside that genus (45). Similarly, *Crepidospermum* and *Tetragastris*

are nested in *Protium* (46) and were merged into that genus. For the small proportion of names whose validity could not be determined with those tools, we used *The Plant List* (47). Lianas, bamboos, tree ferns, and tree-sized herbs were excluded from all analyses. Varieties and subspecies were ignored (i.e., all individuals were assigned to the species level). Although some individuals may be misidentified, we assume that this error is within acceptable limits, especially for common species (see discussion above).

The total number of trees ≥ 10 cm dbh in Amazonia was estimated as follows. First, the study area was divided into 567 1° -grid cells (DGCs; Fig. 1). We constructed a loess regression model for tree density (stems ha^{-1}) on the basis of observed tree density in 1195 plots, with latitude, longitude, and their interaction as independent variables. The span was set at 0.5 to yield a relatively

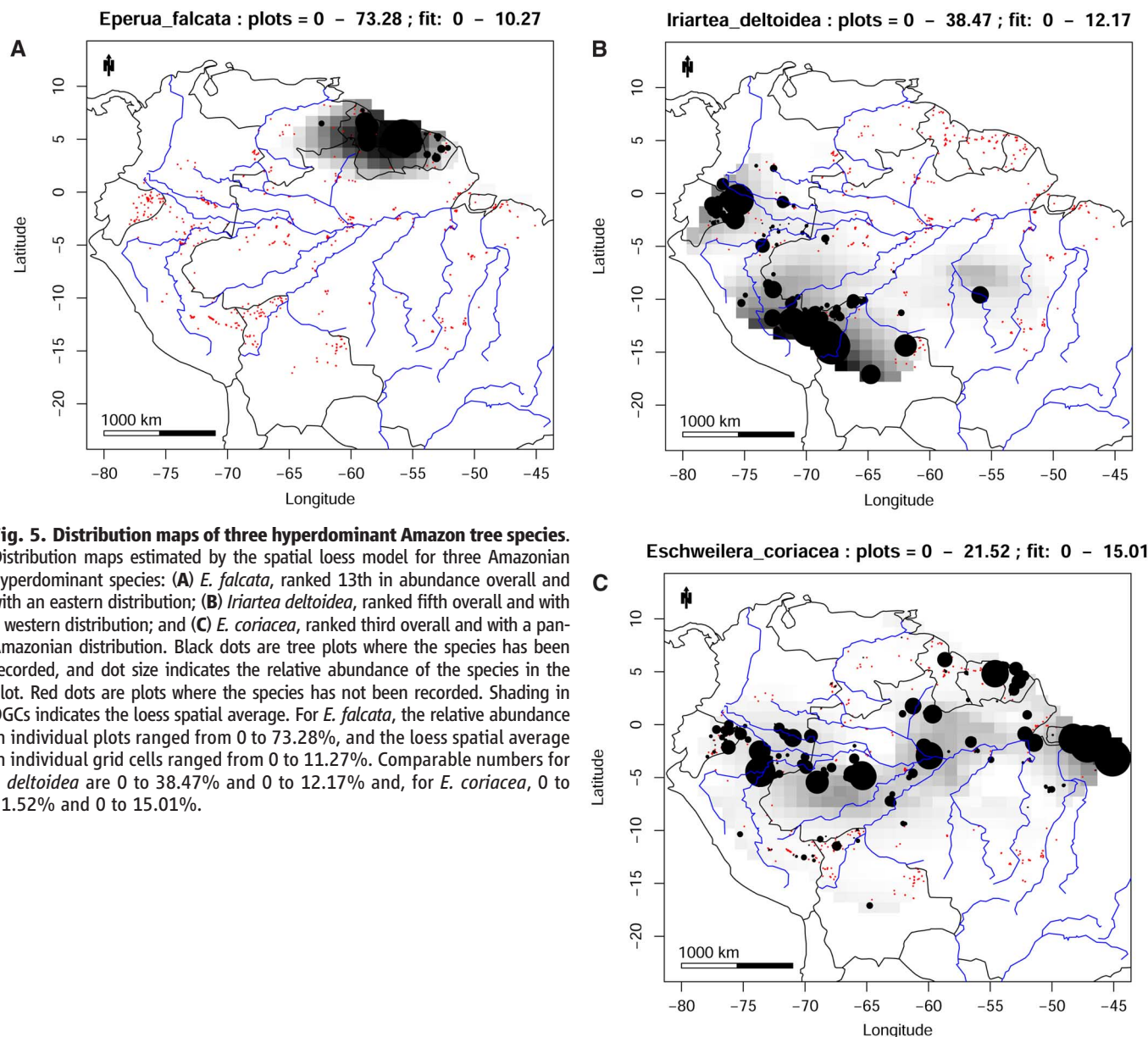


Fig. 5. Distribution maps of three hyperdominant Amazon tree species. Distribution maps estimated by the spatial loess model for three Amazonian hyperdominant species: (A) *E. falcata*, ranked 13th in abundance overall and with an eastern distribution; (B) *Iriarteia deltoidea*, ranked fifth overall and with a western distribution; and (C) *E. coriacea*, ranked third overall and with a pan-Amazonian distribution. Black dots are tree plots where the species has been recorded, and dot size indicates the relative abundance of the species in the plot. Red dots are plots where the species has not been recorded. Shading in DGCs indicates the loess spatial average. For *E. falcata*, the relative abundance in individual plots ranged from 0 to 73.28%, and the loess spatial average in individual grid cells ranged from 0 to 11.27%. Comparable numbers for *I. deltoidea* are 0 to 38.47% and 0 to 12.17% and, for *E. coriacea*, 0 to 21.52% and 0 to 15.01%.

smooth average. The model was used to estimate average tree density in each DGC (D_{DGC} , stems ha^{-1}). The total number of trees in each DGC (N_{DGC}) was then calculated by multiplying D_{DGC} by 1,232,100 ha (the area of a DGC close to the equator—the deviation from this area is just 2.8% at 14°S and 1.1% at 8°N, our latitudinal range). Both empirical (plot data) and interpolated tree densities are illustrated in fig. S4.

The total number of trees belonging to each species in Amazonia was estimated as follows. Abundances of all valid species were converted to relative abundances for each plot: $RA_i = n_i/N$, where n_i = the number of individuals of species i and N = the total number of trees in the plot (including unidentified trees).

For each of the 4962 species with a valid name, we constructed a loess model for RA_i , with latitude, longitude, and their interaction as independent variables and a span of 0.2. We used only spatially independent variables, because test runs including environmental variables commonly led to predictions of species occurrences in well-sampled areas where they had never been recorded in plots. For a similar reason (i.e., to keep predictions spatially conservative), a smaller span was used than in the tree density analysis. Negative predicted abundances were set to 0. The loess model of a species predicted relative abundance in each DGC, yielding a map of its predicted variation in relative abundances across Amazonia. The total population size of each species was calculated by multiplying its relative abundance in each DGC by the total number of trees in that DGC and then summing these products for all DGCs.

To reduce the impact of individual plots and quantify uncertainty in the above procedure, we carried out a bootstrap exercise. This involved randomly drawing 1000 plots from the 1170-plot data set (with replacement) and calculating the population sizes of all species as described above. This was repeated 500 times, and the 500 population estimates per species were used to calculate mean estimated population size and 95% confidence intervals (i.e., mean $\pm$ 1.96 SD).

To estimate range size, we used the same data and methods as (48), standardized with TNRS and updated with specimen records from SpeciesLink (49). Species not found in this database were left out of the range size analysis ($n = 842$). Worldwide species diversity of genera was estimated by counting accepted species in (47). Seed mass and wood density data were obtained from sources described in (36).

Habitat preference was analyzed by means of Indicator Species Analysis, a permutation test that calculates indicator values for each species based on their frequency and relative abundance (50) in the five forest types (igapó, terra firme, swamp, várzea, and white-sand forest).

To analyze regional-level dominance, we divided Amazonia into six regions and created a RAD for each region by summing population sizes in the DGCs they contained. RADs were also constructed for each forest type by sum-

ming the individuals of each species in all plots of a given forest type and calculating the average density of each species in that forest type. The forest-type RADs thus have their basis not in population estimates in DGCs but in the raw abundance data in our plots. A species was considered dominant in a given region or forest type if it appeared in the list of species comprising the upper 50% percentile of the respective RAD.

All analyses were carried out with the R software platform (51). For Indicator Species Analysis, we used the package labdsv. All other permutation tests were custom written.

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Supplementary Materials

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Supplementary Text
Figs. S1 to S12
Tables S1 to S3
Appendices S1 to S4
References (53–67)

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A Complete Skull from Dmanisi, Georgia, and the Evolutionary Biology of Early *Homo*

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The site of Dmanisi, Georgia, has yielded an impressive sample of hominid cranial and postcranial remains, documenting the presence of *Homo* outside Africa around 1.8 million years ago. Here we report on a new cranium from Dmanisi (D4500) that, together with its mandible (D2600), represents the world's first completely preserved adult hominid skull from the early Pleistocene. D4500/D2600 combines a small braincase (546 cubic centimeters) with a large prognathic face and exhibits close morphological affinities with the earliest known *Homo* fossils from Africa. The Dmanisi sample, which now comprises five crania, provides direct evidence for wide morphological variation within and among early *Homo* paleodemes. This implies the existence of a single evolving lineage of early *Homo*, with phylogeographic continuity across continents.

Excavations at Dmanisi, Georgia, have yielded hominid fossils, a rich vertebrate fauna, and Mode I (Oldowan) stone artifacts co-occurring in well-understood geological and taphonomic contexts. Occupation of the site began shortly after 1.85 million years ago (Ma) and is documented until about 1.77 Ma (1–6). The temporal and geographic setting and the preservation of at least five hominid individuals make Dmanisi a site that is crucial in understanding patterns of variation, biogeography, and evolution within emergent *Homo*. The generalized (primitive) craniomandibular morphology, small cranial capacity, moderate body size, and a mosaic of primitive and derived postcranial features link the Dmanisi sample to largely contemporaneous fossils from Africa attributed to *H. habilis* and/or *H. erectus*. Various features of the cranial vault and base also indicate affinities to *H. erectus* from East Asia (4, 5, 7, 8).

Here we present a complete cranium (Fig. 1) that substantially expands the range of variation seen in the Dmanisi sample (Fig. 2), with implications as to the early evolutionary history of the genus *Homo*. Specimen D4500 was recovered in 2005 from the base of layer B1y in Block 2 of the Dmanisi excavation area (fig. S1) (9). D4500 represents the same individual as mandible D2600, found 5 years earlier (10). The anatomical association of D4500 and D2600 (hereafter “skull 5”) constitutes the most complete adult skull known from Early Pleistocene *Homo* (hereafter “early *Homo*”; the definition of Pleistocene follows

the new terminology). Skull 5 is probably associated with the postcranial elements of an adult individual with nearly modern human body proportions [(5); supplementary text S1 and fig. S1]. Because skull 5 is intact and free from taphonomic deformation, it is now possible to document the morphology of an entire adult cranium with its associated mandible and teeth and to study anatomy not previously known for early *Homo*.

Description of Cranium D4500

The morphology of skull 5 stands apart from that of any other known fossil *Homo* specimen through its combination of a small braincase with a large prognathic face (Fig. 1, Table 1, supplementary text S2 and S3, figs. S2 and S3, and tables S1 to S3).

Braincase

The endocranial volume, (ECV) evaluated from computed tomography (CT) data, is $546 \pm 5 \text{ cm}^3$. Coupled with its small ECV, the braincase is low but comparatively wide and basally elongate. The frontal squama is moderately sloping. The parietals are bossed and exhibit only slight midline keeling in their posterior half. The zygomatic arches are massive, and spacious temporal fossae indicate voluminous masticatory muscles. The temporal lines are well separated from each other (minimum distance from cranial midplane 12 mm behind bregma: 20 mm). The temporal bone exhibits prominent supramastoid crests and laterally wide porial saddles, which give the neurocranium a squat, bell-like shape when viewed from the rear.

The upper squama of the occipital bone has a marked protuberance immediately below its lambdoid margin, which extends across the midplane by ~30 mm on either side. The prominence of this “lambdoid hump,” together with a massive protruding inion, lends the upper occipital

plane a nearly vertical orientation, whereas the nuchal plane is only moderately inclined (21°) relative to the Frankfurt horizontal (FH) plane. The occipital transverse torus is ruggedly built, and the nuchal region is deeply sculpted. A bilaminar crest linking inion with opisthion suggests a strong nuchal ligament. The mastoid processes are large and steeply inclined medially. Their inferior portion is compressed mediolaterally to form a distinctive flange-like structure that extends posteriorly. Overall, cranial superstructures of D4500 are massive and more prominent than in the other Dmanisi individuals, suggesting that it represents a male.

Temporal Bones and Cranial Base

The right zygomatic arch exhibits in vivo deformation indicating a healed but displaced multiple fracture behind the masseteric origin. The left temporomandibular joint (TMJ) as well as the left mandibular condyle bear evidence of degenerative arthritic deformation. The right TMJ and left zygomatic arch are unaffected by pathology. There is marked lateral extension of the TMJ, with 61% of the glenoid fossa beyond the external wall of the vault, below the zygomatic shelf. The fossa itself is shallow, transversely oriented (tympanomedian angle 3°), and has a low anterior wall that merges smoothly with the preglenoid planum. The tympanic plate is massive and exhibits a prominent vaginal process. Medially, the tympanic is expanded to form a robust supratubarius process. The petrous pyramid closely approaches the wall of the basioccipital, thus reducing the foramen lacerum to a crevice. The main axis of the pyramid forms an angle of 34° (petromedian angle) with the midsagittal plane. The foramen magnum is oval in shape and level (inclination 0°) with respect to the FH. Basicranial flexion, as measured by the orientation of the clival plane relative to the sphenoid plane, is low (128°).

Face

Contrasting with the small braincase, the face of skull 5 is among the largest and most prognathic known from early *Homo*. The glabellar region is massive and substantially overhangs the midface. The orbits are overarched by thick bar-like supraorbital tori; a shallow post-toral shelf (rather than a sulcus) extends posteriorly toward the frontal squama. Postorbital constriction is marked. The zygomatic processes of the maxillae are massive and laterally flaring. Their anterior surface is vertically oriented and coincides with the orbital plane, thus forming a zygomatico-orbital plane (fig. S2). As a consequence of the tall and wide midface, the zygomatico-alveolar crests are markedly angled. The roots of the zygomatic processes originate between the first and second molars. The nasal floor is delimited anteriorly by a smooth (rather than sharply angled) sill. The inclination of the naso-alveolar clivus relative to the alveolar plane is 42°. The subnasal portion of the clivus

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is slightly concave, whereas its alveolar portion is convex, covering the large incisor roots. The palate is long and arched; the incisive foramen is located at the position of the second premolar (P^4), indicating marked prognathism of the maxilla and anterior dentition.

Dentition

The upper and lower dentition of skull 5 is heavily worn, and crown morphologies are only partially preserved. Estimated molar dimensions are comparatively large, and maxillary as well as man-

dibular molar sizes increase from M1 to M3 (Table 1 and table S3A). Judging from root orientation, the maxillary incisors were strikingly procumbent. The open bite (structural non-occlusion of the incisors), rounded labial wear surfaces on upper incisors and rounded lingual wear surfaces on lower incisors, respectively, and labiolingually oriented striations on these surfaces suggest that the anterior dentition was used in paramasticatory activities such as gripping (11). The first maxillary premolars (P^3 s) are situated posterolaterally to Cs, such that massive C/ P^3

juga square off the front of the maxilla. Similarly massive C/ P^3 juga are present in the mandible D2600 (12). P^3 s have three roots; P^4 s are double-rooted, with the buccal root exhibiting two canals (three-rooted P^3 s and double-rooted P^4 s have also been reported for other early *Homo* specimens; see table S3B). The P^3 s of mandible D2600 have two roots (the distal root with two canals) (12). The P^4 s are not preserved.

Comparative Analysis

Skull 5 is the first complete specimen to provide evidence of how the face (including the mandible) of adult early *Homo* was oriented and positioned relative to the braincase. Hitherto, complete craniomandibular morphology of early *Homo* was represented by two adolescents, whose facial skeletons were not yet fully developed (KNM-WT 15000 and D2700/D2735) (2, 13), and the senile Dmanisi individual D3444/D3900, whose gnathic morphology was strongly modified by alveolar bone resorption after tooth loss (3, 4) (Fig. 2). Among other adult early *Homo* specimens from Africa and East Asia, anatomical connections between preserved neurocranial and maxillofacial parts are typically incomplete, and facial-to-neurocranial orientation remains a matter of debate (14, 15). The current perception of skull morphology of early *Homo* is thus strongly influenced by adolescent representatives (and the less complete and taphonomically distorted adult cranium KNM-ER 1813) that exhibit comparatively orthognathic faces with lightly built superstructures. The architecture of skull 5 now indicates that small-brained, large-faced, remarkably prognathic and robust morphologies all belonged to the normal range of variation of early *Homo*.

The ECV of skull 5 (546 cm³) represents the smallest of the Dmanisi sample (skulls 1 to 4: 601 to 730 cm³), and it is at the lower end of variation of the *H. habilis* hypodigm [509 to 687 cm³ (16, 17)]. Stature and body mass estimates obtained from the postcranial elements that are probably associated with skull 5 [146 to 166 cm, 47 to 50 kg (5)] place this individual within the range of variation estimated for early *Homo* and at the lower end of variation of African *H. erectus* and modern humans (18). The skull 5 individual thus provides the first evidence that early *Homo* comprised adult individuals with small brains but body mass, stature, and limb proportions reaching the lower range limit of modern human variation (5, 18). Combining ECV and body mass data, the encephalization quotient (EQ) of the skull 5 individual is estimated as ~2.4, which is within the range of variation for *Australopithecus* (16). The larger ECV of early *Homo* as compared to *Australopithecus africanus* [340 to 515 cm³ (16)] and *Au. sediba* [420 cm³ (19)] might thus primarily reflect an evolutionary increase in body size rather than increased encephalization.

Although similarities between Dmanisi and eastern African fossils attributed to *H. ergaster* and *H. erectus* have previously been documented

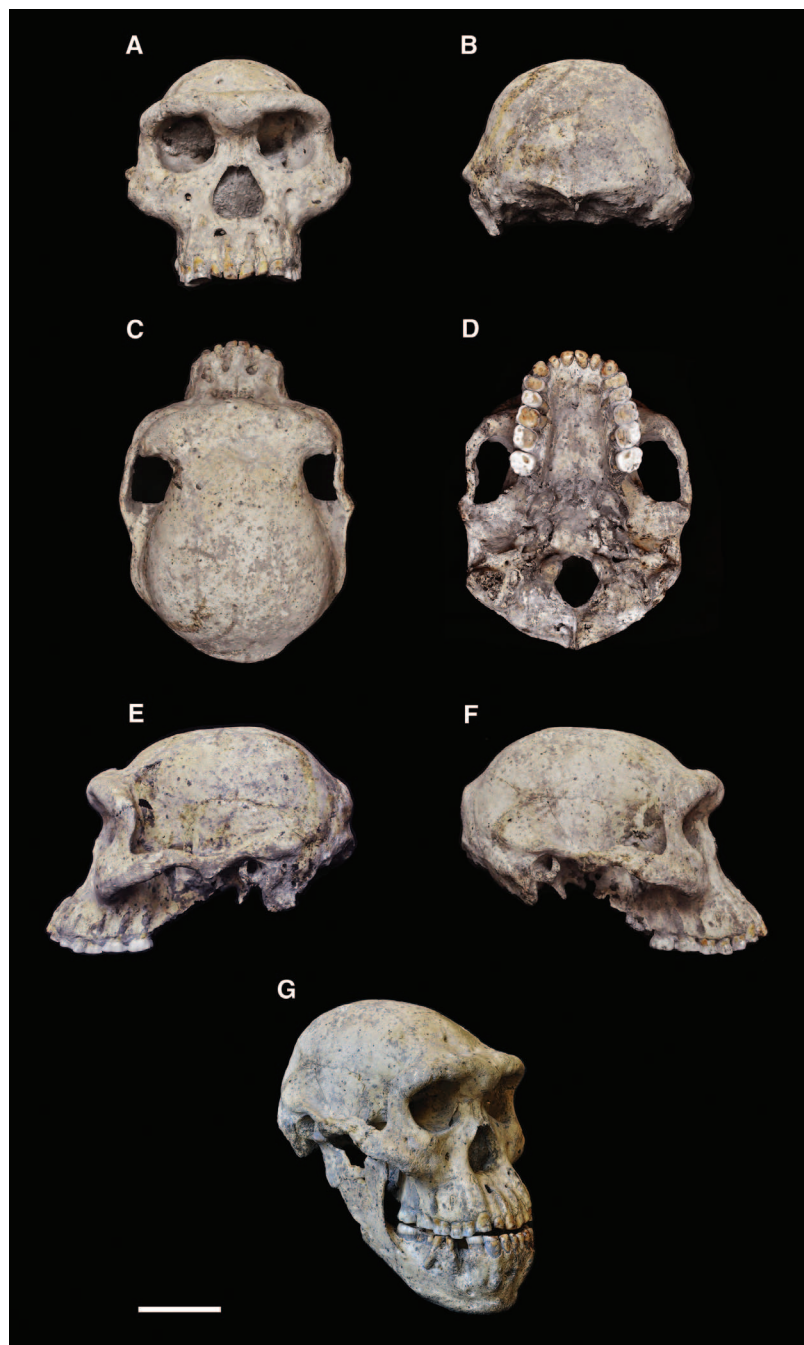


Fig. 1. Dmanisi cranium D4500 and associated mandible D2600. (A and B) Anterior and posterior views; (C and D) superior and inferior views; (E and F) left and right views; (G) cranium with articulated mandible. Scale bar, 5 cm.

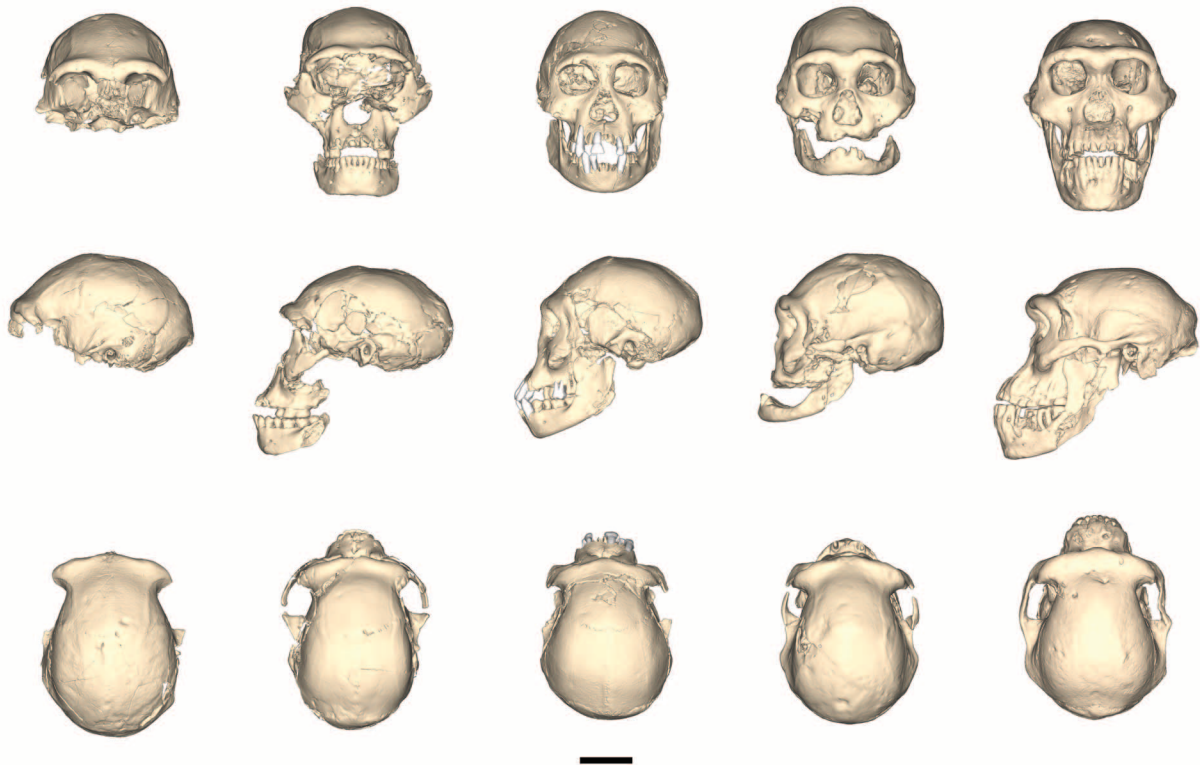


Fig. 2. The Dmanisi paleodeme. CT-based visualizations, from left to right: D2280 (skull 1), D2282/D211 (skull 2), D2700/D2735 (skull 3), D3444/D3900 (skull 4), D4500/D2600 (skull 5). Scale bar, 5 cm. See fig. S3 for a full set of standard views.

(4, 7, 8, 20), skull 5 permits us to widen the scope of comparisons (supplementary text S4 and table S4). In midfacial architecture, skull 5 exhibits morphological affinities with the broadly contemporaneous African specimens SK 847 (fig. S4) and OH 65 (21), as well as with early representatives of *Homo* in eastern Africa such as KNM-ER 1470 and KNM-ER 62000, dated to ~2.0 to 1.9 Ma (15). In all these specimens, the naso-alveolar clivus is moderately sloping and laterally wide, curving around the buccal roots of the P³s.

The Chemeron temporal fragment, dated to 2.4 Ma, was proposed as the earliest fossil evidence of *Homo* (22, 23), but given the wide variation in TMJ position and morphology in the Dmanisi and early *Homo* samples (table S4), this assignment remains inconclusive. However, the maxilla of skull 5 is similar in overall form and dento-alveolar features to the well-preserved early *Homo* maxilla A.L. 666-1 from Hadar, Ethiopia, with a geological age of 2.33 Ma (24) (fig. S4 and table S4). The generic and chronological status of A.L. 666-1 has recently been questioned, and *A. sediba* (1.977 Ma) has been proposed instead as a potential ancestor of the *Homo* lineage (25). The close morphological affinities between A.L. 666-1, additional early *Homo* specimens from eastern Africa such as OH 65 and KNM-ER 1470 (supplementary text S4), and now skull 5, however, effectively falsify such a scenario (26).

Table 1. Craniodental dimensions of skull 5. Linear dimensions are in millimeters, angles in degrees, areas in square millimeters, and volumes in cubic centimeters.

Endocranial volume	546 (541–551)
Glabella-opisthocranium length	165
Basion-bregma height	92
Nasion-bregma length	86
Biporionic breadth	123
Maximum parietal breadth	109
Cranial base angle (CBA4)	128
Minimum frontal breadth	75
Supraorbital torus thickness	12
Upper facial width (bi-frontomolare temporale)	112
Biorbital width (bi-frontomolare orbitale)	99
Bizygomatic width	149
Midfacial width (bi-zygomaxillare)	109
Nasion-prosthion length	73
Nasion-basion length	99
Basion-prosthion length	127
Palate length (orale-staphylion)	75
Palate width (at M2)	35
Naso-alveolar clivus angle	42
Symphyseal height	50
Molar crown area (buccolingual width x mesiodistal width) M ¹ , M ² , M ³	159, 197, 208
Molar crown area (buccolingual width x mesiodistal width) M ₁ , M ₂ , M ₃	160, 186, 217

Variation now established by the enlarged Dmanisi sample is crucial to identifying patterns of variation among and between paleospecies of early *Homo*. Several studies provide evidence for variation along and across a single evolving lineage of early *Homo* (27, 28), with differences between fossil specimens primarily reflecting intraspecific variation at the population level and

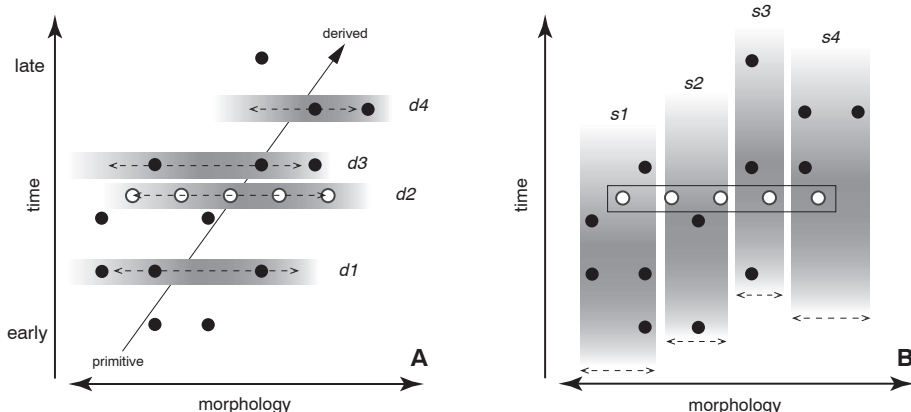


Fig. 3. Two contrasting hypotheses about the evolution of early *Homo*. Hypothesis (A) posits that the fossil record samples paleodemes (horizontal bands, d1 to d4) of a phyletically evolving early *Homo* lineage, each with a separate geographic focus but overlapping in time and morphology. Selection and drift result in an overall morphocline from early generalized to late derived forms (diagonal arrow). This hypothesis is compatible with a wide range of variation within paleodemes of a lineage (dashed double arrows) and is most compatible with the evidence of morphological variation in the Dmanisi deme (white dots). Hypothesis (B) posits that the fossil record [same dots as in (A)] samples distinct paleospecies (vertical bands, s1 to s4) overlapping in time and space. This hypothesis predicts that morphological variation within each lineage is restricted (dashed double arrows), which is incompatible with the combined evidence of multiple contemporary but morphologically disparate crania from Dmanisi (white dots).

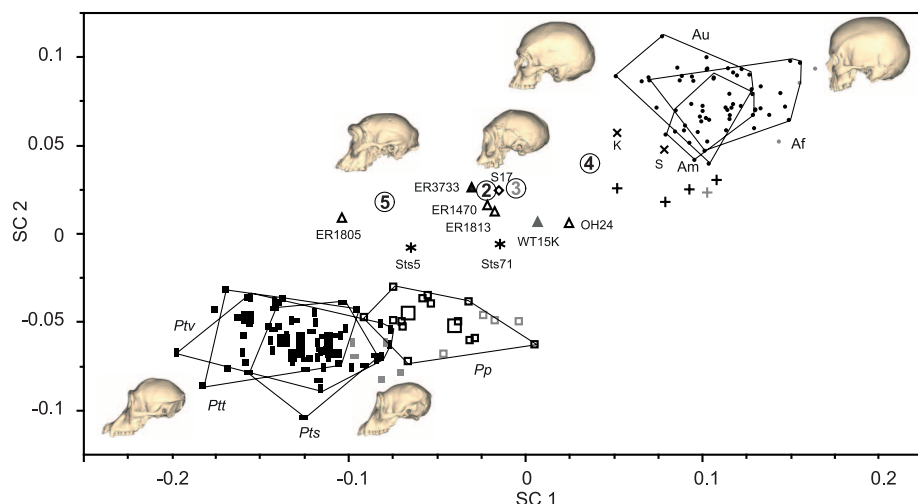


Fig. 4. Shape variation of the Dmanisi crania in comparative context. Dmanisi crania [2:D2282 (skull 2), 3:D2700 (skull 3 and picture), 4:D3444 (skull 4), 5: D4500 (skull 5 and picture)]; African early *Homo* (triangles with specimen names); *A. africanus* (asterisks); *H. erectus* Java (diamond); Kabwe and Steinheim (crosses); *H. neanderthalensis* (plus signs); *H. sapiens* (dots and pictures; populations from Africa, Australia, and America); *P. troglodytes*: *P. t. troglodytes* (solid squares and pictures), *P. t. verus* (vertical rectangles), *P. t. schweinfurthii* (horizontal rectangles); *P. paniscus* (open squares). Black symbols indicate adult individuals; gray symbols indicate subadult individuals. Large symbols indicate male and female averages. Shape component SC1 captures within-group cranial variation from large-faced/prognathic to small-faced/orthognathic individuals; SC2 captures shape change associated with grade shifts in neurocranial size between taxa.

evolutionary change within a species over time (Fig. 3A). Other studies, however, interpret variation in small samples of early African *Homo* as reflecting taxonomic species diversity (15, 29–31) and posit three or more largely contemporary paleo-species (*H. rudolfensis*, *H. habilis*, and *H. erectus*). According to this interpretation (Fig. 3B), mor-

phological differences among individual fossil specimens indicate interspecific differences and concomitant phyletic diversification.

Testing these contrasting hypotheses depends on information about variation within paleodemes; that is, within populations of a fossil species at a given point in space and time (32) (Fig. 3). A

fossil sample is likely to represent a paleodeme if two conditions are met. It must come from a spatially and temporally constrained stratigraphic and taphonomic setting [which is the case for Dmanisi (4, 5)], and within-sample variation must be similar in range and mode to within-deme variation in closely related extant species.

Geometric morphometric analysis and resampling statistics show that craniomandibular shape variation among the Dmanisi hominids is congruent with patterns and ranges of variation in chimpanzee and bonobo demes (*Pan troglodytes troglodytes*, *P. t. verus*, *P. t. schweinfurthii*, and *P. paniscus*) and in a global sample of *H. sapiens* (Fig. 4, supplementary text S5 to S7, and figs. S7 to S9). Within all groups, variation in cranial shape is mainly due to interindividual differences in size and orientation of the face relative to the braincase. The Dmanisi sample, including skull 5, thus represents normal within-deme variation, ranging from small-faced relatively orthognathic (typically female and/or subadult) individuals to large-faced relatively prognathic (typically male) individuals.

Comparative analysis of nonmetric cranial characters also indicates that within-deme variation is substantial and wider than typically recognized (supplementary text S2 to S4 and table S4). The Dmanisi paleodeme shows variation in maxillofacial features, which have been interpreted as evidence for taxonomic diversity in eastern African fossils (15, 31).

The Dmanisi sample thus represents the best currently available example of cranial form and form variation within the early *Homo* lineage. It can tentatively be characterized as follows (figs. S2 and S3): face with flaring, vertically oriented zygomatic processes that are coplanar with the orbital plane; similar upper and midfacial widths; markedly angled zygomaticoalveolar crests; marked alveolar prognathism; C roots exhibiting parallel orientation relative to the cranial midplane; naso-alveolar clivus moderately sloping and laterally delimited by C and/or P³ juga; and ECV greater than ~500 cm³. These features also discriminate early *Homo* from nonrobust *Australopithecus* species, which exhibit a more protruding but less sloping naso-alveolar clivus; inclined maxillary zygomatic processes; moderately curved zygomaticoalveolar crests; and superomedially tapering canine juga, which laterally delimit the anterior surface of the maxilla. Other features of early *Homo*, such as three-rooted premolars, marked postorbital narrowing, a shallow glenoid fossa, and a moderate degree of basicranial flexion, represent plesiomorphic (primitive) character states variably shared with the *Australopithecus* species.

Discussion

As already noted by Darwin, recognizing species diversity comes “at the expense of admitting much variation” within species [(33), p. 51]. Together with data from *Au. afarensis* paleodemes such as A.L. 333 (34–36), Dmanisi adds to the growing evidence that intrademic and intraspecific

variation in Plio-Pleistocene fossil hominids tends to be misinterpreted as species diversity, especially when single fossil specimens from different localities are compared with each other (37). Evidence from skull 5 and the other four Dmanisi specimens indicates that cranial shape variation within early *Homo* paleodemes was similar in mode and range to that seen within modern *Pan* demes. Furthermore, Dmanisi indicates that an important proportion of character state variation in nonmetric features reflects intrademic variation rather than real species diversity. These findings have several implications for the interpretation of morphological diversity in the fossil record of early *Homo*.

When seen from the Dmanisi perspective, morphological diversity in the African fossil *Homo* record around 1.8 Ma probably reflects variation between demes of a single evolving lineage, which is appropriately named *H. erectus*. The hypothesis of multiple independent lineages (paleospecies) (15, 31) appears less parsimonious, especially in the absence of empirical evidence for adaptation to separate ecological niches. The hypothesis of phyletic evolution within a single but polymorphic lineage raises a classificatory but not evolutionary dilemma, and it is premature to describe the rate(s) of evolution in this lineage, given the small available samples. Specimens previously attributed to *H. ergaster* are thus sensibly classified as a chronosubspecies, *H. erectus ergaster*. The Dmanisi population probably originated from an Early Pleistocene expansion of the *H. erectus* lineage from Africa, so it is sensibly placed within *H. e. ergaster* and formally designated as *H. e. e. georgicus* to denote the geographic location of this deme [thus retracting the species status given earlier to mandible D2600 (12)].

Given the scattered and fragmentary fossil record in Africa that predates Dmanisi, questions of earliest African *Homo* phylogenetics and classification remain unresolved. It remains to be tested whether all of the fossils currently allocated to the taxa *H. habilis* and *H. rudolfensis* belong to a single evolving *Homo* lineage. Although we regard this null hypothesis as parsimonious and fully compatible with new evidence from Dmanisi, alternative scenarios exist. Given the range of variation seen in the Dmanisi paleodeme, there is no convincing signature, at present, of early *Homo* cladogenesis. The African fossils that post-date the Dmanisi ensemble show brain size increase and correlated change in craniofacial morphology within the evolving lineage of *H. erectus*. Moreover, it is likely that both the underpinning of the East Asian dispersal of *H. erectus*, as well as the roots of subsequent *H. erectus* evolution in Africa (for example, OH 9, Daka), shared greater craniofacial robusticity.

The new evidence from the ancient Dmanisi deme of early *Homo* reinforces the strong African affinities previously recognized for this early Eurasian outpost of our genus (7). Variation between continents may thus provide insight into

the evolutionary population dynamics of early *Homo*. It is well known from the upper Pleistocene dispersal of modern humans from Africa that genetic and phenetic variation within demes decreases with geographic distance from Africa, due to serial founder effects and population bottlenecks. For example, modern human phenetic variation in western and eastern Asia is 95 and 85% of African variation, respectively (38). The expansion of early *Homo* from Africa might have occurred at longer time scales, so direct comparisons must remain tentative. Nevertheless, the observation that Dmanisi conserves a substantial proportion of cranial shape and its variation among early African *Homo* speaks for genetic continuity between Africa and Eurasia. Because intrademic variation at Dmanisi is similar to that found in extant and extinct near relatives, the effective population size of early *Homo* in western Asia might have been similar [$N_e > 10,000$ (39)]. Furthermore, the remarkably large and robust dentognathic remains of early *H. erectus* from Java (Trinil/Sangiran) (40, 41) exhibit close affinities with skull 5 (tables S2 to S4). This provides evidence for morphological (and presumably underpinning genetic) continuity across large geographic distances, and for the preservation in East Asia of an appreciable proportion of the variation originally present within paleodemes of early *Homo* in Africa (20, 42–44) (fig. S8).

Contrasting with early *Homo* population continuity across continents, current paleontological data indicate a low degree of similarity between contemporaneous mammalian genera in Africa and at Dmanisi (5), implying a generally low rate of faunal exchange between Africa and Asia before and around 1.8 Ma (45). As suggested earlier (46), the populational, ecological, and evolutionary dynamics of early *Homo* probably differed significantly from those of coeval large mammals, including other hominid lineages. Theoretical considerations indicate that the long-range dispersal rate of a population mainly depends on rates of reproduction and local habitat expansion (47) and that intragroup cooperation can play an important role in population persistence (48). Various features such as derived lower limb and foot morphologies (5, 49), tool-mediated widening of the dietary niche toward meat eating (4, 6), and increased levels of intragroup cooperation (3) might have led to increased rates of reproduction, survival, and mobility in early *Homo* and the consequent establishment of stable populations outside Africa. Skull 5 and the other members of the Dmanisi paleodeme now indicate that early *Homo* expanded from Africa to ultimately establish substantial populations in western Asia. This earliest hominid dispersal pre-dated any significant increase in brain size. Further analyses are required to test and modify the attendant hypotheses. Ultimately, identifying hominid paleodemes and assessing within-deme variation (32) will be key to understanding mechanisms of evolution and geographic dispersal of early *Homo*.

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Supplementary Materials

www.sciencemag.org/content/342/6156/326/suppl/DC1
Supplementary Text S1 to S6
Figs. S1 to S9
Tables S1 to S7
References (50–96)

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REPORTS

Stellar Spin-Orbit Misalignment in a Multiplanet System

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Stars hosting hot Jupiters are often observed to have high obliquities, whereas stars with multiple coplanar planets have been seen to have low obliquities. This has been interpreted as evidence that hot-Jupiter formation is linked to dynamical disruption, as opposed to planet migration through a protoplanetary disk. We used asteroseismology to measure a large obliquity for Kepler-56, a red giant star hosting two transiting coplanar planets. These observations show that spin-orbit misalignments are not confined to hot-Jupiter systems. Misalignments in a broader class of systems had been predicted as a consequence of torques from wide-orbiting companions, and indeed radial velocity measurements revealed a third companion in the Kepler-56 system.

The Kepler space telescope detects exoplanets by measuring periodic dimmings of light as a planet passes in front of its host star (*I*). The majority of the ~150,000 targets observed by Kepler are unevolved stars near the main sequence, because those stars provide the best prospect for detecting habitable planets similar to Earth (2). In contrast, the temperature and surface gravity of Kepler-56 (KIC6448890) indicate that it is an evolved star with exhausted hydrogen in its core and that it started burning hydrogen in a shell surrounding an inert helium core. Stellar evolutionary theory predicts that our Sun will evolve into a low-luminosity red giant similar in size to Kepler-56 in roughly 7 billion years.

The Kepler planet search pipeline detected two planet candidates orbiting Kepler-56 (designated as KOI-1241) (3) with periods of 10.50 and 21.41 days, a nearly 2:1 commensurability. The observation of transit time variations caused by gravitational interactions showed that the two candidates represent objects orbiting the same star, and modeling of these variations led to upper limits on their masses that place them firmly in

the planetary regime (4). Kepler-56 is the most evolved star observed by Kepler with more than one detected planet. Transit observations lead to measurements of planet properties relative to stellar properties, and hence accurate knowledge of the host star is required to characterize the system. Asteroseismology enables inference of stellar properties through the measurement of oscillations excited by near-surface convection (5). The power spectrum of the Kepler-56 data after removing the planetary transits shows a regular series of peaks (Fig. 1), which are characteristic of stellar oscillations. By combining the measured oscillation frequencies with the effective temperature and chemical composition obtained from spectroscopy, we were able to precisely determine the properties of the host star (6). Kepler-56 is more than four times as large as the Sun and its mass is 30% greater (Table 1).

Nonradial oscillations in evolved stars are mixed modes, behaving like pressure modes in the envelope and like gravity modes in the core (7, 8). Unlike pressure-dominated mixed modes, gravity-dominated mixed modes have frequencies that are shifted from the regular asymptotic

spacing. Mixed modes are also approximately equally spaced in period (9). We measured the average period spacing between dipole (*l* = 1) modes in Kepler-56 to be 50 s, consistent with a first-ascent red giant (*l*0).

Individual mixed dipole modes are further split into multiplets as a result of stellar rotation. Because the modes in each multiplet are on average expected to be excited to very nearly equal amplitudes, the observed relative amplitudes depend only on viewing angle relative to the stellar rotation axis (*I*). For Kepler-56, several mixed dipole modes show triplets (Fig. 1). A rotation axis perpendicular to the line of sight (inclination *i* = 90°) would have produced a frequency doublet, whereas a star viewed pole-on (*i* = 0°) would have produced no visible splitting (6). Therefore, the observed triplets are a clear signature of an intermediate inclination of the stellar rotation axis with respect to the line of sight. This also implies an intermediate inclination with respect to the planetary

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orbital axes, which are known to be perpendicular to the line of sight from the existence of transits.

For a quantitative measurement of the stellar spin-axis inclination, we modeled the six dipole modes with the highest signal-to-noise values. The fitted parameters included the frequency, height, width, rotational splitting, and inclination for each mode. Three fitted modes (Fig. 1B, top) correspond to gravity-dominated mixed modes, whereas the other three multiplets (Fig. 1B, bottom) are pressure-dominated mixed modes. The best-fitting model yields an inclination angle $i_g = 43^\circ \pm 4^\circ$ for gravity-dominated modes and $i_p = 51^\circ \pm 4^\circ$ for pressure-dominated modes. Simulations confirmed that the inclination measurements are not strongly affected by the stochastic

excitation of the oscillation modes, and both inclinations are consistent with the coarser determination that can be made from estimates of the spectroscopic projected rotational velocity and the surface rotation rate (6). Furthermore, the measured splittings for gravity-dominated mixed modes are substantially higher than for pressure-dominated mixed modes, consistent with internal differential rotation in red giant stars (12). Our observations thus reveal that Kepler-56 rotates differentially with a rapidly spinning core and that both the core and the envelope are (within 1.46) mutually aligned and inclined by about 45° to the line of sight of the observer.

To measure the properties and orbital parameters of the planets, we used the stellar properties

from asteroseismology to fit a model to the Kepler data that includes gravitational interactions between the planets, as revealed in the transit time variations (a “photodynamical” model; Fig. 2). In addition to the Kepler light curves, we obtained 10 high-precision radial velocity measurements using the High-Resolution Echelle Spectrometer (HIRES) at the Keck 10-m telescope. The radial velocities show the Doppler signal of the transiting planets, as well as a slow velocity drift indicating a third, more massive companion in a wide orbit (Fig. 3). The combined fit of transit time variations and radial velocity data yields precise properties of the system (Table 1). Both planets have densities consistent with gas-giant planets, and their radii are comparable to the

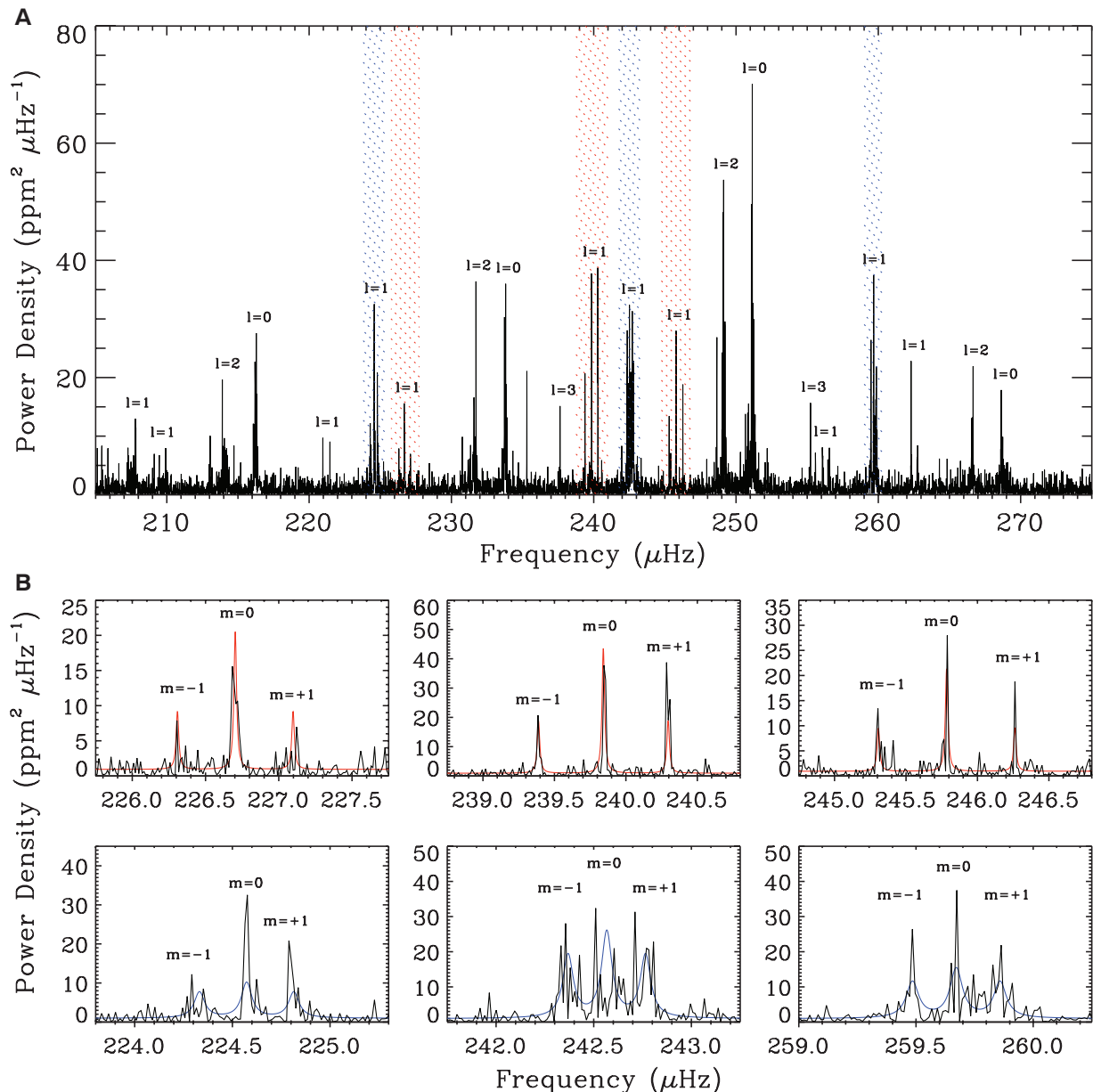


Fig. 1. Power spectrum analysis to measure the inclination of the stellar rotation axis. (A) Power spectrum centered on the frequency range with excited oscillations. The spherical degree l of each identified mode is indicated. Red and blue areas highlight gravity-dominated and pressure-dominated mixed

dipole modes, respectively. (B) The six mixed dipole modes highlighted in (A). Each mode is split into a triplet by rotation. The azimuthal order m of each component is indicated. Red and blue lines show the modeled Lorentzian profiles. The scatter in the data about the fitted model is due to the finite mode lifetimes (6).

radius of Jupiter R_J for planet c ($R = 0.88 \pm 0.04 R_J$) and intermediate between the radii of Saturn and Neptune for planet b ($R = 0.58 \pm 0.03 R_J$). The planets are more than 30% larger than previously thought (4) because asteroseismology enables

a more accurate measurement of the host star's properties. Further analysis also shows that the orbits of the planets are nearly circular and coplanar. By itself the pattern of transit time variations

does not imply coplanar orbits, but in combination with the radial velocity data, the mutual inclination is required to be either very low ($< \sim 10^\circ$) or moderately high ($> \sim 60^\circ$) (6). We performed dynamical stability simulations using initial conditions drawn from the posterior distribution of the photodynamical model. The highly inclined solutions were dynamically unstable on a time scale of 10^4 years (6). Thus, the transiting planets in the Kepler-56 system are on coplanar orbits that are misaligned with the equatorial plane of the host star.

Several theories have been proposed to explain stellar spin-orbit misalignments. Favored scenarios include dynamical perturbations such as Kozai cycles (13) and planet-planet scattering (14). These scenarios would be consistent with the presence of a third companion, but would tend to randomize mutual inclinations of planets (and therefore lead to mutually inclined multi-planet systems) unless the perturbations occurred early enough for the inclinations to be damped by the protoplanetary disk. Alternative tilting mechanisms invoke interactions of the stellar magnetic field with the protoplanetary disc (15), angular momentum transport within the star by internal gravity waves (16), or tidal interactions in the early stages of star formation (17). These theories are consistent with a coplanar multi-planet system but do not require the presence of a third companion on a wide orbit. Spin-orbit misalignments could also be produced through a scenario involving torques from nearby planets or companion stars in inclined orbits (18, 19). Contrary to other scenarios, such a mechanism would naturally produce both a coplanar multi-planet system and a third companion in a wide orbit, as observed for Kepler-56.

The wide companion in the Kepler-56 system thus offers an intriguing explanation for the misalignment based on a scenario originally proposed for the transiting multiplanet system HAT-P-13 (18). The radial velocity drift implies a third companion with the mass of a gas-giant planet within a few astronomical units, or a brown dwarf or star within several dozen astronomical units. In either case, if the third companion's orbit is itself inclined with respect to the inner planetary orbits (for example, through planet-planet scattering if the companion is a planet), it could have torqued the orbits of the inner planets out of the equatorial plane of the host star. The inner planetary orbits would stay aligned with one another because of strong coupling between their orbits, resulting in a misalignment of the two coplanar transiting planets with the host star. Dynamical simulations that include a third companion in an eccentric orbit inclined to the equatorial plane of the host star confirm that such a mechanism can reproduce the architecture of the Kepler-56 system (6).

Obliquity measurements have long been considered as a powerful tool to test planet formation theories (20, 21). In particular, observations of the Rossiter-McLaughlin effect have revealed that stars hosting hot Jupiters (mass $> \sim 0.3 M_J$,

Table 1. Properties of the Kepler-56 system. Host star properties were derived using asteroseismology and high-resolution spectroscopy. The inclination angle was calculated as a weighted average of the inclination measured from gravity-dominated and pressure-dominated dipole modes and includes uncertainties from finite mode lifetimes (6). Because the orbits are not periodic, orbital periods and transit times for the planets refer to values at an arbitrary reference epoch [barycentric Julian date (BJD) 2,454,970]. The mutual orbital inclination of the two planets is $5_{-3.1}^{+3.4}$ degrees at this epoch.

Host star	
Radius ($R_\odot$)	4.23 ± 0.15
Mass ($M_\odot$)	1.32 ± 0.13
Mean density (g cm^{-3})	0.0246 ± 0.0006
log[Surface gravity (cgs)]	3.31 ± 0.01
Effective temperature (K)	4840 ± 97
Metallicity [M/H] (dex)	0.20 ± 0.16
Age (billions of years)	3.5 ± 1.3
Stellar inclination (degrees)	47 ± 6
Planet b	
Time of transit (BJD)	$2454978.2556_{-0.0057}^{+0.0056}$
Orbital period (days)	$10.5016_{-0.0010}^{+0.0011}$
Semimajor axis (AU)	$0.1028_{-0.0037}^{+0.0037}$
Radius ($R_\oplus$)	$6.51_{-0.29}^{+0.29}$
Mass ($M_\oplus$)	$22.1_{-3.6}^{+3.9}$
Mean density (g cm^{-3})	$0.442_{-0.072}^{+0.080}$
Planet c	
Time of transit (BJD)	$2454978.6560_{-0.0055}^{+0.0057}$
Orbital period (days)	$21.4023_{-0.00062}^{+0.00059}$
Semimajor axis (AU)	$0.1652_{-0.0059}^{+0.0059}$
Radius ($R_\oplus$)	$9.80_{-0.46}^{+0.46}$
Mass ($M_\oplus$)	181_{-19}^{+21}
Mean density (g cm^{-3})	$1.06_{-0.13}^{+0.14}$

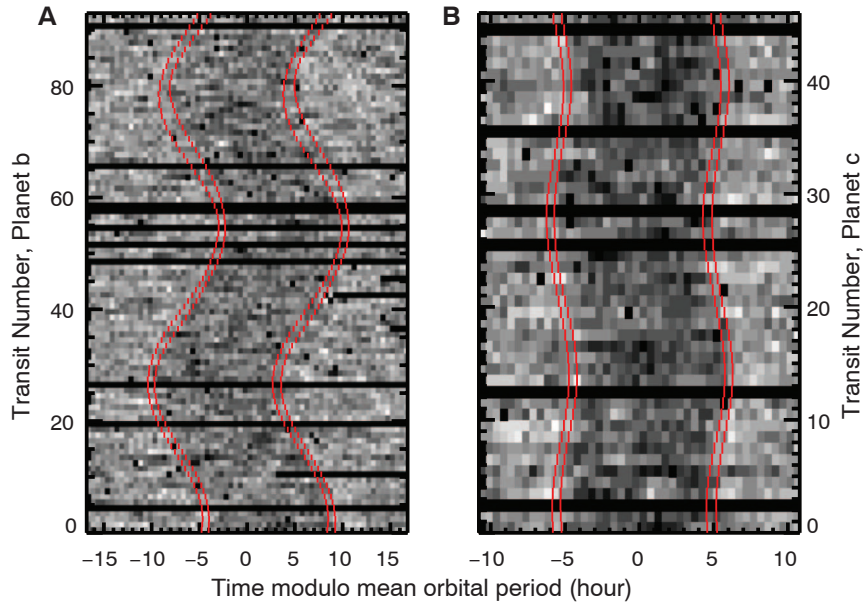


Fig. 2. Transit time variations of the inner planets. Stellar intensity is plotted as a function of transit epoch and time modulo the mean orbital period near transits of planet b (A) and planet c (B). The red lines mark the 68% confidence intervals for the start and end of each transit according to the photodynamical model.

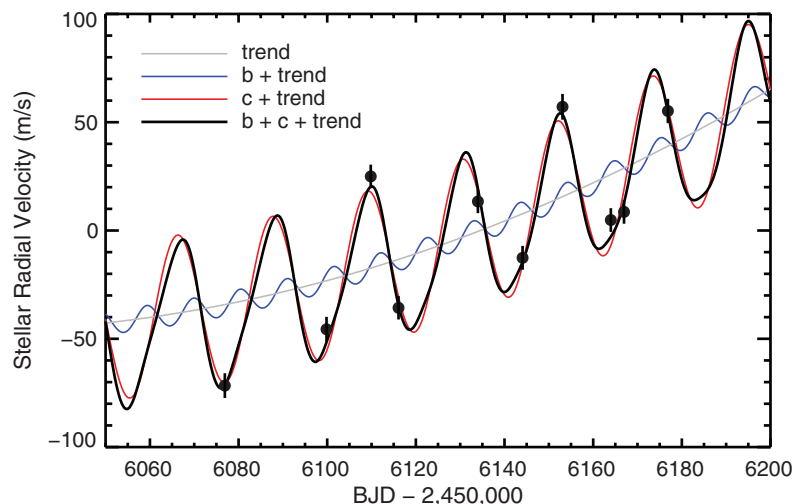


Fig. 3. Radial velocity variations. Solid circles show the individual radial velocity measurements as a function of barycentric Julian date (BJD); the black solid line is the best-fitting photodynamical model to the combined Kepler and radial velocity data. Thin gray, blue, and red lines show the individual components of the fit, which includes a radial velocity drift modeled as a quadratic function of time and radial velocity variations due to planets b and c. The drift is attributed to a third, massive companion in a wide orbit.

period < 10 days) show a wide range of obliquities (22–25). This finding has been interpreted as supporting evidence for dynamical perturbations as the origin of hot Jupiters, and against scenarios in which hot Jupiters migrate inward because of an interaction with the protoplanetary disk (26). This conclusion, however, relies on the assumption that the stellar equator is a good tracer of the initial orbital plane of the planet (and hence the protoplanetary disk), which has previously been called into question (27, 28). Important test cases are coplanar multiplanet systems, which, if primordial alignments are common, should predominantly show low obliquities. Indeed, until now all transiting multiplanet systems have been found to be well-aligned (29–31).

Although our observations do not constrain the primordial inclination of the protoplanetary disk of Kepler-56, they provide firm evidence that stellar spin-orbit misalignments are not solely confined to hot-Jupiter systems. Continued radial velocity measurements will reveal whether the third companion in the Kepler-56 system is a planet (implying that the initial misalignment occurred after the planets formed) or a star (implying a primordial misalignment of the protoplanetary disk).

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Supplementary Materials

www.sciencemag.org/content/342/6156/331/suppl/DC1
Materials and Methods
Figs. S1 to S24
Tables S1 to S6
References (32–119)

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Gravitational-Wave Limits from Pulsar Timing Constrain Supermassive Black Hole Evolution

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The formation and growth processes of supermassive black holes (SMBHs) are not well constrained. SMBH population models, however, provide specific predictions for the properties of the gravitational-wave background (GWB) from binary SMBHs in merging galaxies throughout the universe. Using observations from the Parkes Pulsar Timing Array, we constrain the fractional GWB energy density (Ω_{GWB}) with 95% confidence to be $\Omega_{\text{GWB}}/H_0/73$ kilometers per second per megaparsec² < 1.3×10^{-9} (where H_0 is the Hubble constant) at a frequency of 2.8 nanohertz, which is approximately a factor of 6 more stringent than previous limits. We compare our limit to models of the SMBH population and find inconsistencies at confidence levels between 46 and 91%. For example, the standard galaxy formation model implemented in the Millennium Simulation Project is inconsistent with our limit with 50% probability.

Supermassive black holes (SMBHs), with masses between 10^6 and 10^{11} solar masses, are observed to exist at the centers of all

massive galaxies in the nearby universe and to have masses that scale closely with properties of their hosts (1, 2). Together, these phenomena

suggest that the growth processes of SMBHs and of their host galaxies are connected. Galaxies, and groups of galaxies, are embedded in even larger dark matter halos, which form and evolve through the hierarchical merging of smaller dark matter halos and galaxies (3, 4). Galaxy mergers are expected to result in binary SMBHs (5, 6), which, though notoriously difficult to observe via electromagnetic signatures, are expected to be the strongest sources of gravitational waves in the universe (7). The universality of galaxy mergers implies the existence of a gravitational-wave background (GWB) from binary SMBHs (8, 9).

The GWB is manifested as a red-noise process in pulse arrival time measurements from pulsars (10). Pulsar timing array groups search for evidence of the GWB in radio-frequency observations of millisecond pulsars, which have rivaled the stability of the best clocks on Earth over time scales of tens of years (11). The GWB is commonly parameterized by its wave amplitude spectrum, $h_c(f) = A(f/f_{\text{yr}})^{-2/3}$, where f is the received gravitational-wave frequency, f_{yr} is a reference frequency of one cycle per year, and A is the characteristic amplitude that defines the strength of the GWB. The fraction of the critical energy density of the universe, per logarithmic frequency interval (Ω_{GW}), of the GWB is $\Omega_{\text{GW}}(f) = (2\pi^2/3H_0^2)A^2f_{\text{yr}}^2(f/f_{\text{yr}})^{2/3}$ (10), where H_0 is the Hubble constant, which we assume to be $73 \text{ km s}^{-1} \text{ Mpc}^{-1}$. Recent observations by two separate pulsar timing array groups have been analyzed to find $A < 6 \times 10^{-15}$ (12) and $< 7 \times 10^{-15}$ (13) with 95% confidence.

We have been monitoring pulse arrival times from 20 millisecond pulsars with the 64-m Parkes Telescope as part of the Parkes Pulsar Timing Array (PPTA) project (14) and previous observing programs (15). We extended the timing baseline of this data set by including publicly available observations from the Arecibo observatory (16). A detection of the GWB relies on measuring correlations between residual pulse arrival times for

multiple pulsars with different angular separations on the sky. Within the PPTA timing program, there are presently too few pulsars with sufficient timing precision and data span to make an unambiguous detection of the GWB feasible (17). We instead use observations of six pulsars with the lowest noise levels over the longest observing spans to constrain the GWB amplitude (18) (Fig. 1).

Our limit on the strength of the GWB was computed in two stages (19). For each pulsar j , we first estimated the power spectral density $P_j(f_i)$ of the residual pulse arrival times after a fit for a pulsar model (20) at frequencies f_i that are harmonics of $1/T_{\text{obs}}$, where T_{obs} is the observing span for the pulsar. A prewhitening method (21) was used in the spectral estimation to eliminate spectral leakage and to provide nearly independent spectral estimates, even if red-noise signals,

such as those expected from the GWB, are present. We form a detection statistic (DS) from the power spectra

$$A^2 = \sum_i [P_j(f_i)g_j(f_i)/M_j(f_i)^2] / \sum_i [g_j(f_i)/M_j(f_i)]^2 \quad (1)$$

where $g_j(f_i)$ is the shape of the power spectrum induced by the GWB and $M_j(f_i)$ is a model of the observed spectrum (Fig. 1). The DS A^2 combines individual spectral estimates $P_j(f_i)$ to form a conservative estimate of the square of the characteristic amplitude of the GWB (A^2). If the spectral models are correct, a DS of the form in Eq. 1 provides an estimate of A^2 with a maximal signal-to-noise ratio. To set a limit on A , we compared the observed value of the DS with distributions of the

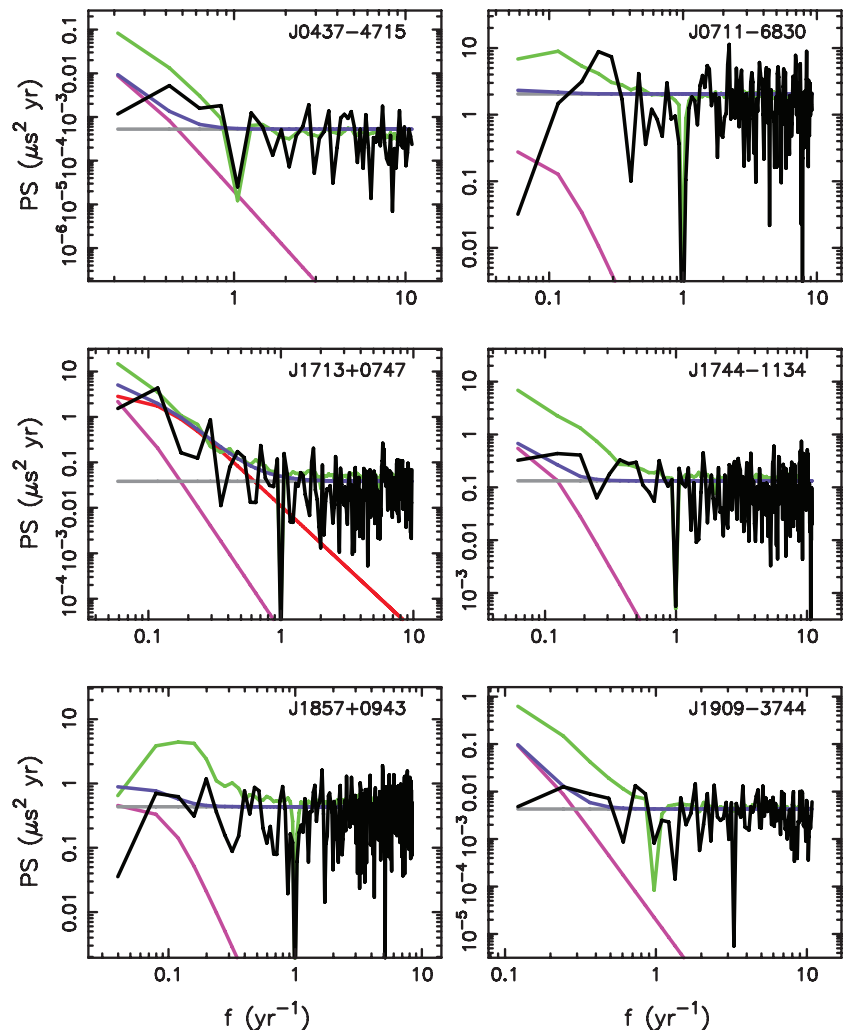


Fig. 1. Observed power spectra of the residual postfit arrival times and models of these spectra for the PPTA pulsars used to set the limit on the GWB amplitude. The observed power spectra (PS), P_j , for the pulsars are shown as black lines, along with the models of the PS, $M_j = W_j + G_j + R_j$ (shown as purple lines). The models contain a white component (W_j , gray lines), a common GWB component G_j (pink lines), and, for PSR J1713+0747, an additional red-noise term R_j (red line). The PS models were used only for the determination of the weights in the calculation of the detection statistic. The green curves show what the PS would look like (on average) in the presence of a Gaussian GWB with amplitude 2.4×10^{-15} . The names of the pulsars are given in the top-right corners of each panel.

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DS derived from simulated data sets, which include white noise consistent with the observations and a GWB of strength A_{sim} . Many trial simulations were conducted at a given A_{sim} to account for the stochasticity of the GWB. The 95% confidence limit on the GWB amplitude, A_{95} , is the value of A_{sim} at which only 5% of the A^2 trials are lower than the observed A^2 .

We simulated both Gaussian (10) and non-Gaussian (9) GWB-induced residual pulse arrival times. Although previous pulsar timing array limits on the strength of the GWB (12, 13) were derived assuming Gaussian statistics, a non-Gaussian background, dominated by fewer binary SMBHs, is predicted from some models of the binary SMBH population (8, 9).

We verified the efficacy of the algorithm by correctly bounding the GWB strength in synthetic data sets, including those in the International Pulsar Timing Array Data Challenge and other mock data sets that contained features of the observations such as inhomogeneous observing cadence, highly heteroscedastic pulse arrival times, and red noise (22). When applied to the PPTA data set, and assuming a Gaussian GWB, we find that $\Omega_{\text{GW}}(f_{\text{PPTA}})(H_0/73 \text{ km s}^{-1} \text{ Mpc}^{-1})^2 < 1.3 \times 10^{-9}$ with 95% confidence at a gravitational-wave frequency (f_{PPTA}) of 2.8 nHz (23). This is equivalent to $A_{95} = 2.4 \times 10^{-15}$. Compared with the power spectra P_j of the measured residual pulse arrival times, the mean power spectra of 200 simulated realizations with $A_{\text{sim}} = A_{95}$ (displayed in Fig. 1 as green lines) show, as expected, excess power at the lowest frequencies. For a non-Gaussian GWB, we find $\Omega_{\text{GW}}(f_{\text{PPTA}})(H_0/73 \text{ km s}^{-1} \text{ Mpc}^{-1})^2 < 1.6 \times 10^{-9}$ with 95% confidence, corresponding to $A_{95} = 2.7 \times 10^{-15}$.

The PPTA bound on the GWB enables direct tests of models for galaxy and SMBH formation that specify the population of binary SMBHs in the universe. We compared the probability $Pr(\Omega_{\text{GW}})$ that a GWB of energy density $\Omega_{\text{GW}}(f_{\text{PPTA}})$ exists, given the PPTA observations with four predictions for the GWB from binary SMBHs, expressed as the probability density function of $\Omega_{\text{GW}}(f_{\text{PPTA}})$, $\rho_M(\Omega_{\text{GW}})$ (24) (Fig. 2). All four predictions account for the most recent SMBH mass and galaxy bulge mass measurements and include the assumption that all binary SMBHs that contribute to the GWB are in circular orbits and not interacting with their environments.

First, a model that assumes a scenario in which all evolution in the galaxy stellar mass function and in the SMBH mass function is merger-driven at redshifts $z < 1$ (25) predicts a Gaussian GWB that is ruled out at the 91% confidence level. However, the assumption of purely merger-driven evolution leads to the largest possible GWB amplitude, given observational data.

A synthesis of possible combinations of current observational estimates of the galaxy merger rate and SMBH-galaxy scaling relations results in a large range of possible GWB amplitudes (26). PPTA observations exclude 46% of this set of GWB amplitudes, assuming a Gaussian GWB.

As a specific example for how pulsar timing array observations can affect models of SMBH formation and growth, we calculated the level of $\Omega_{\text{GW}}(f_{\text{PPTA}})$ (24) expected from a semi-analytic galaxy formation model (4) implemented within the Millennium (27) and Millennium-II (28) dark matter simulations. This model, in which SMBHs are seeded in every galaxy merger remnant at early times and grow primarily by gas accretion triggered by galaxy mergers, represents the standard paradigm of galaxy and SMBH formation and evolution. The model accurately reproduces the luminosity function of quasars at $z < 1$ corresponding to the epoch predicted to dominate the GWB (8, 25, 26). The range of predictions for $\Omega_{\text{GW}}(f_{\text{PPTA}})$ results from the finite observational sample of measured SMBH and bulge mass pairs (2), which is used to tune the model, but neither accounts for uncertainties in the observed galaxy stellar mass function (4) nor in the nature of the relations between SMBH masses and bulge masses (2). Assuming a non-Gaussian GWB, the probability that this prediction for $\rho_M(\Omega_{\text{GW}})$ will be inconsistent with the PPTA data is 49%.

A complementary prediction for the strength of the GWB comes from an independent model for SMBH growth at late times (29). This model examines the growth mechanisms of SMBHs in cluster and void environments through mergers and gas accretion. The model is inconsistent with the PPTA data at the 61% confidence level.

The PPTA constraints on the GWB show that pulsar timing array observations have reached a sufficient level of sensitivity to test models for the binary SMBH population. The highest galaxy merger rate that is consistent with the observed evolution in the galaxy stellar mass function (25) is inconsistent with our limit. We exclude 46% of the parameter space of a model that surveys empirical uncertainties in the growth and merger of galaxies and black holes (26); therefore, our results reduce these uncertainties. Although the PPTA limit excludes only 49 and 61% of realizations of the GWB from two galaxy and SMBH evolution models, these models are open to refinement. For example, these models do not include SMBH formation mechanisms consistent with high-redshift quasar observations (30), nor do

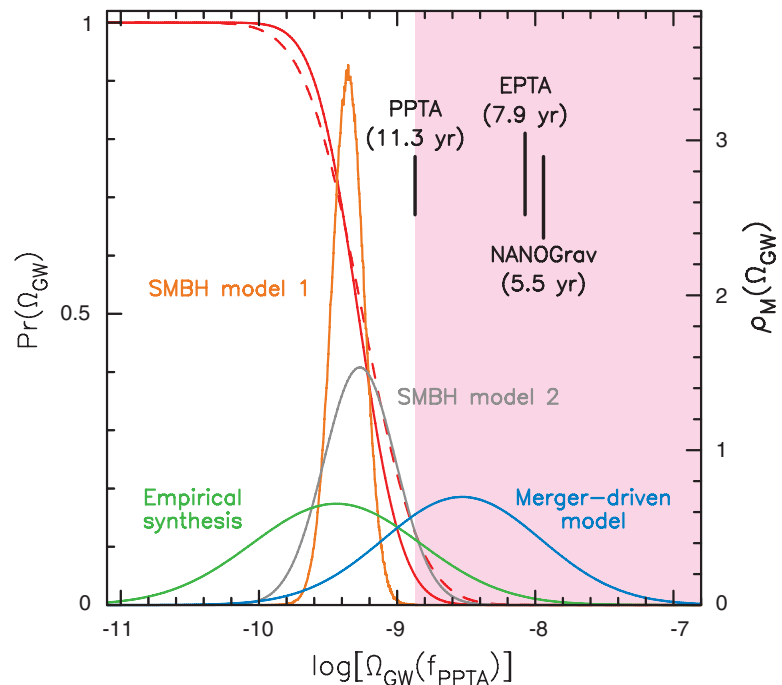


Fig. 2. Comparison between the PPTA constraints on $\Omega_{\text{GW}}(f_{\text{PPTA}})$ and various model predictions (24). Given the PPTA data, the probabilities $Pr(\Omega_{\text{GW}})$ that a GWB relative energy density $\Omega_{\text{GW}}(f_{\text{PPTA}})$ exists, assuming Gaussian (10) and non-Gaussian (9) GWB statistics, are shown as red solid and dashed lines, respectively. The pink shaded area represents the values of $\Omega_{\text{GW}}(f_{\text{PPTA}})$ ruled out with greater than 95% confidence, assuming a Gaussian GWB. The labeled curves represent the probability density functions $\rho_M(\Omega_{\text{GW}})$ for $\Omega_{\text{GW}}(f_{\text{PPTA}})$ predicted by a synthesis of empirical models (26) (green), assuming merger-driven galaxy evolution at redshifts $z < 1$ (25) (blue), from the semi-analytic galaxy formation model (SMBH model 1, orange) that we discuss in the text and from a second distinct model (29) for SMBH growth (SMBH model 2, gray). When integrated over Ω_{GW} , the product of $Pr(\Omega_{\text{GW}})$ and $\rho_M(\Omega_{\text{GW}})$ gives the probability of the model being consistent with the data. The vertical bars indicate the 95% confidence upper limits on $\Omega_{\text{GW}}(f_{\text{PPTA}})$, assuming a Gaussian GWB from the PPTA, and recently published limits from the European Pulsar Timing Array (EPTA) (12) and the North American Nanohertz Observatory for Gravitational Waves (NANOGrav) (13) scaled to f_{PPTA} . The times next to the limits correspond to the reciprocal of the frequency of maximum sensitivity and are approximately the observing span of the data sets (12, 13, 23).

they reproduce the observed larger scatter and possibly higher normalization in SMBH-galaxy scaling relations for the most massive SMBHs (*1, 2*). Other physical effects will also be built into the next generation of GWB models. For example, recent numerical simulations of massive galaxy mergers predict binary SMBHs with eccentricities ranging between 0.1 (*31*) and 0.9 (*32*). If binaries radiating gravitational waves at frequencies relevant to pulsar timing arrays are considerably eccentric or predominantly evolving under environmental interactions (*33*), the spectral shape of $\Omega_{\text{GW}}(f)$ may differ from current predictions (*34*).

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Supplementary Materials

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Supplementary Text
Fig. S1
Tables S1 and S2
References (35–51)

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Strain-Induced Ultrahard and Ultrastable Nanolaminated Structure in Nickel

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Heavy plastic deformation may refine grains of metals and make them very strong. But the strain-induced refinement saturates at large strains, forming three-dimensional ultrafine-grained (3D UFG) structures with random orientations. Further refinement of this microstructure is limited because of the enhanced mobility of grain boundaries. Very-high-rate shear deformation with high strain gradients was applied in the top surface layer of bulk nickel, where a 2D nanometer-scale laminated structure was induced. The strongly textured nanolaminated structure (average lamellar thickness of 20 nanometers) with low-angle boundaries among the lamellae is ultrahard and ultrastable: It exhibits a hardness of 6.4 gigapascal—which is higher than any reported hardness of the UFG nickel—and a coarsening temperature of 40 kelvin above that in UFG nickel.

Metals can be strengthened by introducing more grain boundaries (GBs) via grain refinement (*1, 2*) or alternatively by generating more dislocations (*3*). Remarkable hardening in metals and alloys induced through heavy plastic deformation originates from both mechanisms: Grains are refined to the sub-micrometer or nanometer scales, and stored

dislocation density is pumped up by orders of magnitude. However, as the strains exceed values between 5 and 30, saturation in grain refinement leads to steady-state three-dimensional (3D) ultrafine-grained (UFG, sub-micrometer-sized) structure with random orientations (*4–6*). Further increases in strain, however, can lead to grain coarsening and dislocation annihilation (*7, 8*). As

grain size drops into the submicro- or nanoscale, GB migration becomes so pronounced that grains coarsen even at ambient temperature (*9*). Because the dislocation multiplication from straining is balanced by the dislocation annihilation due to GB migration, strain-induced structural evolution ceases, and dislocation density reaches its limit, which in heavily deformed metals is typically $\sim 10^{15} \text{ m}^{-2}$.

Among various processing parameters of plastic deformation, strain rate, deformation temperature, and strain gradient seem most relevant to dislocation multiplication and, hence, to structure refinement or reduction of boundary spacing, which governs strength and other mechanical properties of materials. Deformation at high strain rates or at low temperatures may suppress dislocation annihilation kinetics and facilitate forming more GBs or dislocation boundaries. For instance, when nickel (Ni) is compressed at a rate

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of 10^2 and 10^3 s^{-1} to a strain of 2.9, a smaller boundary spacing with a higher dislocation density is formed relative to that under low-rate (10^{-2} s^{-1}) deformation, such as cold rolling and torsion, at comparable strains (10, 11). The fraction of low-angle boundaries becomes larger at higher strain rates (11, 12). Lowering deformation temperature may elevate stored dislocation density and reduce grain sizes in deformed copper (Cu) (12, 13). Strain gradient in plastic deformation is proportional to the density of stored geometrically necessary dislocations (14). A very high density of dislocations (1.8×10^{16} to $3.6 \times 10^{16} \text{ m}^{-2}$, which is one order of magnitude higher than the dislocation density limit in deformed Cu) is observed in thin shear bands in the nanotwinned structure in Cu, owing to the very high strain gradients (12).

In this work, a very-high-rate shear deformation with high strain gradients was applied in the top surface layer of a pure bulk Ni sample, where nanometer-thick laminated (NL) structures were induced. A pure Ni rod [purity of 99.88 weight percent (wt %)] of 10 mm in diameter was subjected to surface mechanical grinding treatment (SMGT) (Fig. 1A) at ambient temperature (15, 16), in which the rod surface layer is plastically sheared with gradient distributions of applied strain rates and strains. In the top surface layer of $\sim 80 \mu\text{m}$ thick, the estimated strain rate varies from 10^3 to 10^4 s^{-1} (topmost surface) to 10^2 to 10^3 s^{-1} ($80 \mu\text{m}$ deep from the treated surface), with corresponding accumulative equivalent strains of 30 to ~ 15 . Strain gradients in the top $80 \mu\text{m}$ vary from 0.4 to $0.1 \mu\text{m}^{-1}$, which is 10 times greater than that in the high-pressure torsion process (17). After the treatment, a depth-dependent gradient microstructure was formed (Fig. 1B): a nanostructured layer in the top surface of $\sim 80 \mu\text{m}$ thick with characteristic sizes below 100 nm , an UFG layer in a depth span of 80 to $\sim 140 \mu\text{m}$, and a deformation layer at $140 \mu\text{m}$ $\sim 1 \text{ mm}$ deep with various strain-induced defects, including dislocations and subboundaries. Deeper than 1 mm is deformation-free substrate.

The UFG layer is characterized by roughly equiaxed 3D grains (with an aspect ratio of <2), with an average size of $\sim 230 \text{ nm}$ and random orientations (Fig. 1, C and D). A large fraction of high-angle GBs ($>80\%$) was noticed from misorientation distribution measured by means of electron back-scattering diffraction (EBSD) and convergent beam electron diffraction (CBED) [in a transmission electron microscope (TEM)] (15). These are the typical characteristics of the steady-state UFG structures of Ni processed via heavy plastic deformation (4, 5, 18). The observed grain sizes and misorientation distribution agree quantitatively with those in the high-pressure torsion Ni samples with a strain of ~ 10 , which is comparable with the estimated accumulative strains in this section (~ 6 to ~ 14).

The topmost few micrometers of thickness are characterized by randomly oriented equiaxed nanograins with an average size of $\sim 11 \text{ nm}$. Deeper than

$10 \mu\text{m}$ are elongated nanosized grains (parallel to the sample surface), with an average transversal size of $18 \pm 5 \text{ nm}$ in cross-sectional TEM observations. With increasing depth from 10 to $50 \mu\text{m}$, no obvious change is seen for transversal sizes of the nanograins, but their aspect ratios increase markedly from a few to greater than 100. Most grains are several micrometers in length, whereas some are broken into sub-micrometer-long segments (Fig. 1, E and F). Similar morphologies were seen in the longitudinal-section TEM images, but with smaller aspect ratios (ranging from a few to ~ 15) (Fig. 1E) than the cross-sectional. Clearly, the subsurface layer in a depth span of 10 to $\sim 50 \mu\text{m}$ consists of 2D laminated grains, a kind of layer-shaped nanostructures (19). Rather uniform thickness is seen across the depth span of 10 to $\sim 50 \mu\text{m}$, ranging from 5 to 50 nm , averaging $20 \pm 7 \text{ nm}$ (Fig. 1E). Such NL structures are distinct from the steady-state UFG structures (Fig. 1C) from heavy plastic deformation.

In a depth span of 50 to $\sim 80 \mu\text{m}$ is a transition layer from the NL structures to the 3D UFG or nanograined (NG) structures, characterized by alternative layers consisting of slightly elongated nanograins (with an average transversal size of 70 nm) and NL structures. Thicknesses of both layers are roughly comparable.

The elongated diffraction spots in the selected area electron diffraction pattern (SAED) indicate a strong texture in the NL structures (Fig. 1E).

High-resolution TEM (HRTEM) observations were performed in order to identify the structure characteristics of boundaries between adjacent lamellae. Shown in Fig. 2A is a typical HRTEM observation of four lamellae (I to IV) along the $\langle 110 \rangle$ zone axis. From the fast Fourier transformation (FFT) (Fig. 2A, inset), one can see a small misorientation of $\sim 7^\circ$ along the $\langle 110 \rangle$ zone axis existing among these lamellae. HRTEM images showed that one of the closely packed $\{111\}$ planes of II (Fig. 2C) is inclined by 7° to that of III (Fig. 2D), which is in accordance with the splitting of (111) spots by an angle of 7° in the FFT. A zoom-in observation of the boundary (Fig. 2B) showed an inclination by 3° between the $\{111\}$ planes in II and III, implying an orientation gradient existing within the nanolamellae. Plenty of HRTEM images showed most boundaries in the NL structures exhibit similar misorientations along a certain axis, ranging from 1° to 8° .

Texture of the NL structures was determined with EBSD in a sample partially recovered at 540°C for 1 hour in order to improve the index rate while retaining the deformation texture. The $\{111\}$ pole figure (Fig. 2E) and orientation distribution function maps (fig. S2) showed that the texture components are major $\{100\}\langle 011 \rangle$ and minor $\{111\}\langle 112 \rangle$ and $\{111\}\langle 110 \rangle$ —typical torsion textures (pure shear) in FCC metals (20). The presence of the strong shear textures (with a

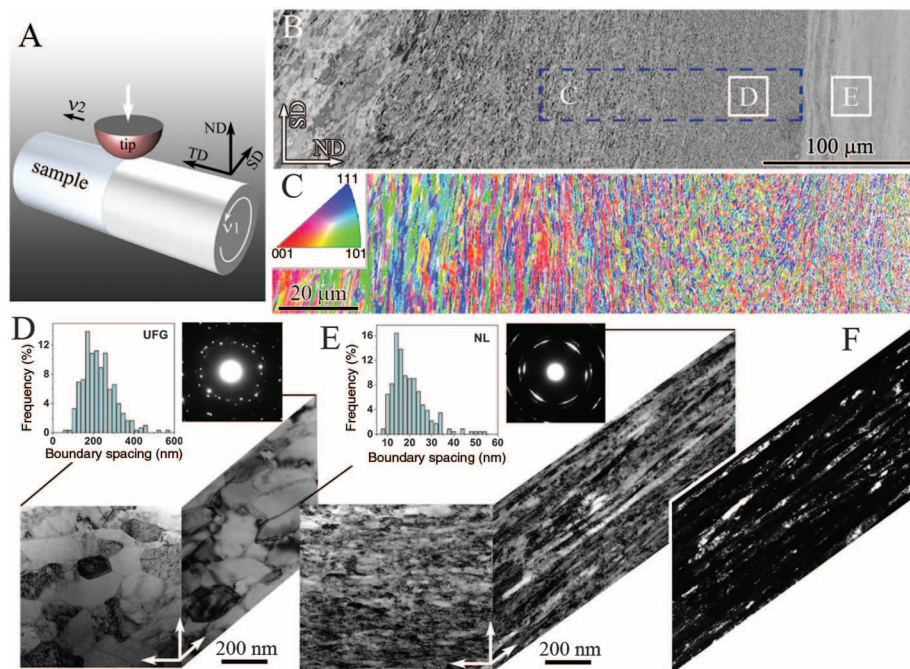


Fig. 1. Processing and microstructure overview of the Ni sample. (A) A schematic illustration of the SMGT setup. (B) A cross-sectional scanning electron microscope image of the SMGT Ni sample. The treated surface is outlined by a yellow dashed line. (C) An EBSD image of the region outlined by dashed blue line in B. (D and E) Typical bright-field cross-sectional (SD-ND) and longitude-sectional (TD-ND) TEM images of the UFG structures ($110 \mu\text{m}$ deep from the surface) and NL structures (40 to $50 \mu\text{m}$ deep from the surface) [indicated in (B)], respectively. Insets in (D) and (E) are the corresponding distribution of boundary spacing (left) and the SAED pattern (right). The sample coordinates in (D) and (E) are identical to that in (A). (F) A dark-field longitude-sectional TEM image corresponding to that in (E).

maximum intensity of ~ 6), which is consistent with the elongated SAED spots (Fig. 1E), indicates heavy shear deformation in the top

surface layer during SMGT processing and operative dislocation activities in the 2D NL structures (21).

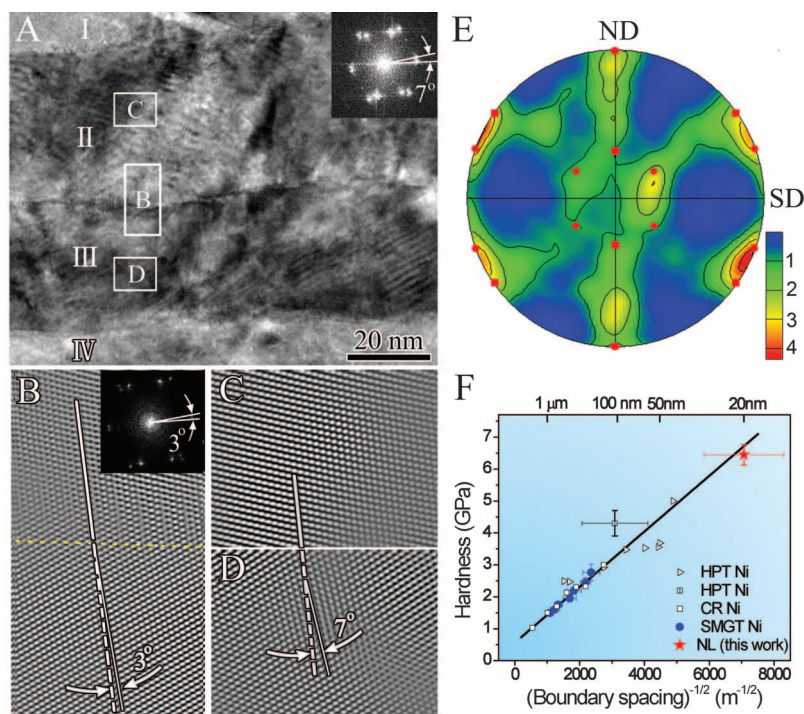


Fig. 2. Boundary structure, texture, and hardness of the NL structures. (A) A HRTEM image of the NL structures containing four lamellae (labeled as I, II, III, and IV). (Inset) A FFT image of lamellae II and III. (B) A Fourier-filtered image shows a contiguity of (11-1) atomic plane in lamella "II" to (111) in lamella "III," with a misorientation angle of 3° across the II/III boundary (yellow dashed line). (Inset) The FFT image also shows superimposition of two {111} planes, with a small misorientation angle. (C and D) Lattice images of lamella "II" and "III," showing that one of the closely packed {111} planes of II (2C) is inclined by 7° to III (2D). (E) {111} Pole figure of the NL structures after annealing at 540°C for 1 hour. The idea orientations of two main shear texture components are indicated with solid squares, $\{100\}\langle 011\rangle$; and solid circles, $\{111\}\langle 112\rangle$. (F) Hall-Petch plot for the UFG and NG structures in Ni samples processed via different techniques reported in the literature (10, 18, 22). The measured hardness of the NL structures (red star) is included for comparison by taking the lamellar thickness equivalent to grain size.

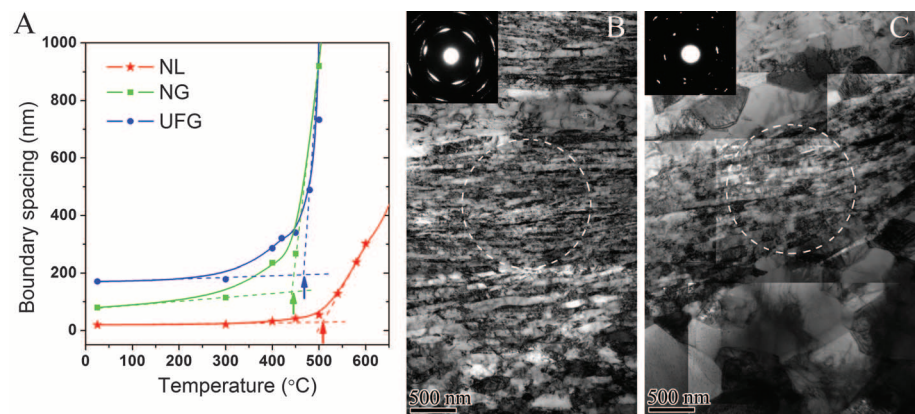


Fig. 3. Thermal stability of the NL structure. (A) Variations of boundary spacing of the UFG, NG, and NL structures as a function of annealing temperature (for 1 hour). (B) A bright-field TEM image of the alternative NG/NL structures at a depth of $\sim 80\ \mu\text{m}$ in the SMGT Ni sample. (C) A bright-field TEM image of the alternative NG/NL structures in the SMGT Ni sample after annealing at 500°C for 1 hour. (Inset) SAED patterns obtained from the respective dashed-line circled regions.

Vickers hardness measurements in the cross-sectional samples indicated that in the UFG layer in a depth span of 80 to $\sim 600\ \mu\text{m}$, hardness varies from 2.76 ± 0.26 to 1.40 ± 0.05 GPa corresponding to different structural sizes. These results are consistent with the reported data for pure Ni (10, 18, 22) and the Hall-Petch relation (Fig. 2E). Measured hardness of the NL structures at 10 to $\sim 50\ \mu\text{m}$ deep averaged 6.4 ± 0.32 GPa. This value is much higher than that in Ni samples processed via plastic deformation reported in the literature (5, 10, 11, 18) and is comparable with that of the electrodeposited Ni with grain sizes of $< 20\ \text{nm}$ (23). Taking lamellar thickness of the NL structures as the equivalent grain size, we found that its hardness lies on the extrapolated Hall-Petch line (Fig. 2E). Such an agreement suggests that the NL boundaries are as effective in resisting dislocation motion as the conventional high-angle GBs. In fact, previous studies showed that contribution of low-angle boundaries to strength is comparable with that of high-angle GBs in strain-induced NG metals (11, 24). In addition, presence of substructures inside lamellae [such as dislocations and twin boundaries as revealed in HRTEM observations (fig. S3)] may provide additional hardening.

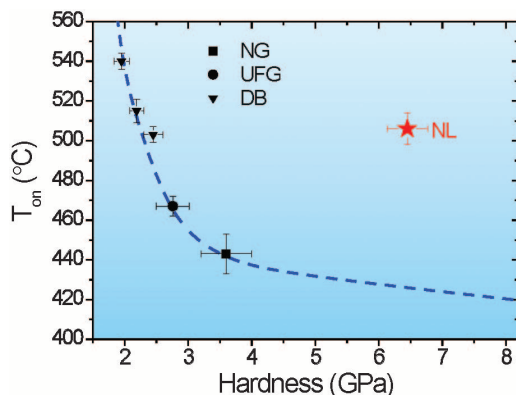
Dislocations are mainly presented at the low-angle boundaries in deformed metals, whereas the dislocation density within the volumes between the boundaries is one or two orders of magnitude lower and hence can be neglected (10, 11, 24). The dislocation density in low-angle boundaries (ρ_{LAB}) is calculated by $\rho_{\text{LAB}} = f_{\text{LAB}} S_v \rho_A$, where f_{LAB} is the fraction of low-angle boundaries, S_v is the boundary area per unit volume, and ρ_A is the dislocation density per unit area of boundary [for a mixed tilt and twist boundary $\rho_A = 1.5\theta/b$ (10, 24), θ is the average misorientation angle of low-angle boundaries and b is the Burgers vector]. With the measured parameters (table S1), a dislocation density of $\sim 1 \times 10^{15}\ \text{m}^{-2}$ was obtained for the UFG structures (120 μm deep from the surface), which is in accordance with the measured result ($\sim 2 \times 10^{15}\ \text{m}^{-2}$) in a torsion-deformed Ni at a comparable strain (25). For the NL structures (40 μm deep from the surface), dislocation density is $\sim 2.4 \times 10^{16}\ \text{m}^{-2}$, which is 20 times greater than that in the UFG. With this dislocation density, hardness of the NL structures can be calculated in terms of the Taylor hardening mechanism by taking it as 3 times the yield stress (10, 24)

$$H_v = 3(\sigma_0 + M\alpha Gb\sqrt{\rho_{\text{LAB}}}) \quad (1)$$

where σ_0 the frictional stress, M is the Taylor factor, α is a constant, and G is the shear modulus. The calculated hardness is 6.7 GPa, which is very close to the measured result (6.4 GPa), verifying the estimated dislocation density.

Thermal stabilities of these microstructures were examined by measuring boundary spacing under TEM after isothermal annealing at 300°C to

Fig. 4. Correlation of thermal stability and hardness. The onset temperature (T_{on}) versus hardness for various microstructures [UFG, NG, and dislocation boundaries (DBs)] in the SMGT Ni at different depths. Solid triangles denote DBs at depths of ~150, 200, and 300 μm from the treated surface.



900°C for 1 hour: 3D UFG structures at ~100 μm deep, 3D NG structures (average size of ~70 nm) at 60 to ~70 μm deep, and 2D NL structures. As in Fig. 3A, no obvious increment in the boundary spacing was detected for these structures after annealing below 300°C. The onset temperature for obvious grain coarsening (T_{on}) is ~443°C for the NG structures and is slightly higher for the UFG (467°C). This is in accordance with the general trend that coarsening temperature decreases for smaller grains (26). For the NL structures, it is surprising to see a much higher onset temperature for lamella thickening of ~506°C. This instability temperature is in agreement with that for the dislocation boundaries in deep layers of the sample (varying from 500° to 540°C, depending on the boundary spacing). The comparable T_{on} of the dislocation boundaries and the NL boundaries implies their similarities in structure.

To verify this observation, the as-SMGT sample was annealed at 500°C (for 1 hour), in between T_{on} of the 3D NG and the 2D NL structures, followed by TEM observations of the alternative NG/NL structures at a depth of ~80 μm . It is seen from Fig. 3, B and C, that the original NG structures transformed into micrometer-sized dislocation-free equiaxed grains—a typical recrystallization product—whereas the NL structures, apart from the slightly increased lamellar thickness due to a recovery process, retained their morphologies without obvious recrystallization. The 2D NL structures with a lamellar thickness of 20 nm are thermally more stable than are the 3D NG structures with grain sizes of 70 nm. The combination of greater hardness and thermal stability in the 2D NL structure is shown in Fig. 4.

No contamination was detected in the sub-surface layer deeper than 5 μm , under the resolution limits of an electron probe microanalyzer (15). The higher thermal stability of the 2D NL structures relative to the 3D UFG and NG structures may be attributed to the low excess energy and low mobility of the low-angle boundaries (27), as well as the extremely large radius of the 2D lamellae, which considerably reduces the capillary force for boundary migration. In addition, the orientation pinning due to the strong textured

lamellae (28), as well as the suppressed recrystallization nucleation kinetics by the laminated structure in extremely small dimensions (29), may also contribute to the elevated stability.

Formation of the NL structures is reasonably attributed to the large shear deformation with very high rates and large strain gradients in the top surface layer of the SMGT sample, which is distinct from the conventional heavy plastic deformation processes such as high-pressure torsion (HPT) and cold rolling. High strain rates promote dislocation multiplication relative to their annihilation (11, 30). The much larger strain gradients in the top surface layer of the SMGT sample than that in the HPT process (typically ~0.04 μm^{-1}) means a much higher geometrically necessary dislocation density is generated because it is proportional to the strain gradient (14). Formation of more low-angle boundaries in form of nanoscale laminated structures is energetically favorable, with an increased density of stored dislocations for minimizing the overall excess energy. Nanoscale laminated structures have also been observed in shear bands that are locally sheared at high rates with large strain gradients in UFG iron samples after compressive deformation (31) and in the nanotwinned Cu during dynamic compression (12). Most boundaries in the sheared regions (or shear bands) in the Cu are of low angle, with an average misorientation of ~7° (12). These observations, in accordance with our present study, suggest that formation of 2D NL structures with a very high dislocation density is a general scenario for strain-induced grain refinement in extremely small dimensions deformed at high strain rates with large strain gradients.

By generating such ultrahard and ultrastable NL structures in surface layers of engineering materials, enhancement in their wear resistance and fatigue properties is anticipated that are sensitive to the surface hardness and structure stability. The very high stored energy of the NL structure may elevate diffusion kinetics and chemical reactivity, facilitating surface modification of engineering alloys. With the simple and cost-effective processing technique, this nanostructure will find potential technological applications in a wide range of industrial manufacturing processes.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 and S2
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Electron-Hole Diffusion Lengths Exceeding 1 Micrometer in an Organometal Trihalide Perovskite Absorber

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Organic-inorganic perovskites have shown promise as high-performance absorbers in solar cells, first as a coating on a mesoporous metal oxide scaffold and more recently as a solid layer in planar heterojunction architectures. Here, we report transient absorption and photoluminescence-quenching measurements to determine the electron-hole diffusion lengths, diffusion constants, and lifetimes in mixed halide ($\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$) and triiodide ($\text{CH}_3\text{NH}_3\text{PbI}_3$) perovskite absorbers. We found that the diffusion lengths are greater than 1 micrometer in the mixed halide perovskite, which is an order of magnitude greater than the absorption depth. In contrast, the triiodide absorber has electron-hole diffusion lengths of ~ 100 nanometers. These results justify the high efficiency of planar heterojunction perovskite solar cells and identify a critical parameter to optimize for future perovskite absorber development.

Photovoltaic (PV) solar energy conversion has the potential to play a major role in future electricity generation. Most currently installed PV arrays consist of crystalline or polycrystalline silicon; in second generation thin-film architectures, light is absorbed and charge generated in a solid layer of this semiconductor (1). Beyond these existing technologies are a myriad of other emerging device concepts based on a broad range of materials, all vying to become the cheapest yet suitably efficient technology to enable widespread uptake of solar energy (2–6). Some of the most promising technologies for ultimate low-cost manufacture are solution-processed, such as organic photovoltaics (OPV), dye-sensitized solar cells (DSSCs), and semiconductor-sensitized

or extremely thin absorber solar cells (7, 8). However, these designs typically require a complex distributed donor-acceptor heterojunction to ionize charge because the exciton and charge carrier diffusion lengths in these materials are much shorter than the absorption depth (7, 9–11). Emerging from the field of DSSCs, the inorganic-organic perovskite family of materials taking the form ABX_3 ($\text{A} = \text{CH}_3\text{NH}_3^+$; $\text{B} = \text{Pb}^{2+}$; and $\text{X} = \text{Cl}^-$, I^- , and/or Br^-) has within the past 12 months been used to fabricate high-performance hybrid solar cells, with reported power conversion efficiencies (η) of between 7 and 15% (12–20). These perovskite absorbers can be solution-processed in air and absorb light broadly across the solar spectrum. In the first experiments, the perovskite absorber was used as a sensitizer on mesoporous titania electrodes, along with a solid-state organic hole transporter (HTM) such as 2'-7,7'-tetrakis(*N,N*-di-*p*-methoxyphenylamine)-9,9'-spirobifluorene (spiro-OMeTAD), with the perovskite effectively replacing the dye conventionally used in a DSSC

(12, 13, 21). Subsequently, the mixed halide $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$ perovskite devices have been reported to yield $\eta > 12\%$ when the titania is replaced with an insulating mesoporous alumina scaffold in meso-superstructured solar cells (MSSCs), demonstrating that the perovskite itself can at least sustain sufficient electron transport to enable highly efficient charge collection (12, 16). Entirely removing the mesoporous alumina to create a thin-film planar heterojunction has resulted in solution-processed devices with close to 100% internal quantum efficiency for charge collection, but with lower η of 5% (16). Very recently, we have demonstrated that if an extremely uniform solid perovskite absorber film can be prepared—for example, through vapor deposition—then the highest efficiencies can be achieved in solid thin-film planar heterojunction architectures (19).

Despite the rapid increase in efficiency associated with the evolution of this technology, most of the fundamental questions concerning the photophysics and device operation remain unanswered. Arguably, the most critical question concerning whether mesostructured or planar heterojunction perovskite solar cells will eventually dominate is what the exciton or the electron and hole diffusion lengths are in these materials. We performed photoluminescence (PL)–quenching measurements in order to extract the electron-hole diffusion lengths in triiodide ($\text{CH}_3\text{NH}_3\text{PbI}_3$) and mixed halide ($\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$) perovskite thin films. We show that both electron and hole diffusion lengths are $> 1 \mu\text{m}$ for the mixed halide perovskite—a factor of ~ 5 to 10 greater than the absorption depth. In contrast, the diffusion lengths in the triiodide perovskite are only on the order of or slightly shorter than the absorption depth ($\sim 100 \text{ nm}$). The larger diffusion length in the mixed halide perovskite results from a much longer recombination lifetime and is consistent with far superior performance in MSSCs and planar heterojunction solar cells, as we demonstrate here.

PL quenching has been previously used successfully with organic semiconductors in order

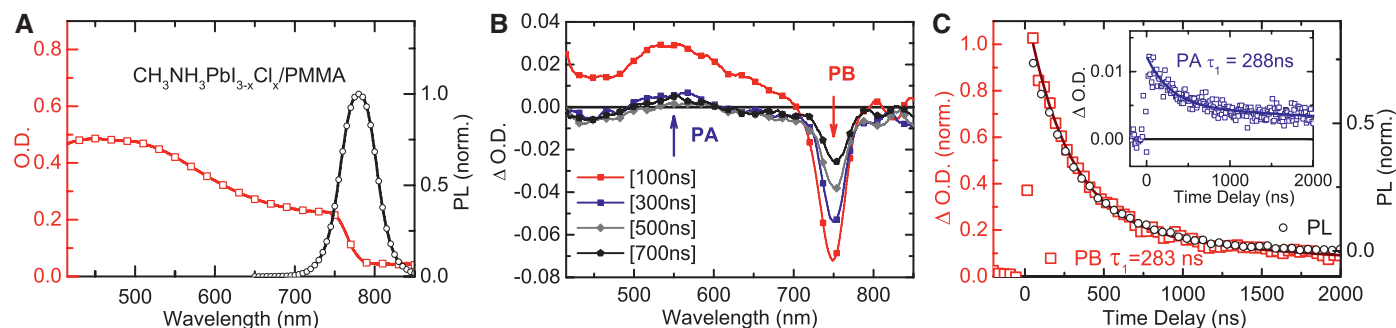


Fig. 1. Optical characterization of the mixed halide perovskite $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$. (A) Absorption (red squares) and PL (black circles) spectra of a 270-nm-thick layer of $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$ coated with PMMA. (B) Transient absorption spectra of $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$ upon excitation at 500 nm ($40 \mu\text{J}/\text{cm}^2$ pulses). Each gated spectrum has been integrated for 200 ns, and the PL was removed. (C) Normalized photobleaching (PB) (red squares, left axis) and PL dynamics (black

circles, right axis) probed at 750 and 770 nm, respectively. Biexponential fitting of the PB data (dark red line) leads to a dominant time constant of $\tau_1 = 283 \pm 6 \text{ ns}$, matching the dynamics of the PL ($\tau_e = 273 \pm 7 \text{ ns}$), followed by a long-lived tail. (Inset) The photoinduced absorption (PA) dynamics at 550 nm (blue squares) with a biexponential fit (dark blue line; dominant time constant of $\tau_1 = 288 \pm 12 \text{ ns}$), also matching the dynamics of the PL and the dominant component of the PB.

to determine the diffusion length of the photo-excited bound electron-hole pair, the exciton (22). By simply fabricating solid thin films in the presence or absence of an exciton-quenching layer, and modeling the PL decay to a diffusion equation, it is possible to accurately determine the exciton lifetime, diffusion rate, and diffusion length (22). However, for the organolead trihalide perovskites studied here, there is relatively little literature on probing the fundamental photophysics, and even the most basic question of whether PL occurs via free carrier recombination from the conduction and valence band electrons, or is preceded by exciton formation, remains unknown. Exciton binding energies for $\text{CH}_3\text{NH}_3\text{PbI}_3$ have been reported in the range of 37 to 50 meV in the orthorhombic phase (23–25). In principle, at ambient temperature these values are comparable to thermal energies ($k_B T \sim 26$ meV, where k_B is the Boltzmann constant and T is the temperature); thus, both free carriers and weakly bound excitons should coexist with interchange being possible between the two populations. However, provided all species (bound or free charges) decay with the same rate or through the same channel, the PL decay should still be representative of recombination or depopulation of electrons and holes from the perovskite film.

The $\text{CH}_3\text{NH}_3\text{PbI}_3$ and $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$ perovskite precursor solutions were spin-coated on plasma-etched glass at room temperature in air, followed by annealing in air at 100°C for 45 min for the mixed halide and 150°C for 15 min for the triiodide (these temperatures and times correspond to the optimized conditions for best performance in the solar cells processed in air). Obtaining uniform and continuous perovskite films is essential for the subsequent PL-quenching measurements. As we have shown elsewhere, this is possible through precise control of processing conditions (26). In order to obtain air- and moisture-insensitive samples, the neat perovskite films (nonquenching samples) were sealed by spin-coating a layer of the insulating polymer poly(methylmethacrylate) (PMMA) on top. Full experimental details are given in the supplementary materials.

In Fig. 1A, we show the ultraviolet-visible absorption and PL spectra for a $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$ thin film. The PL is right at the band edge, with very little Stokes-shift. This indicates little vibronic relaxation of the perovskite crystal, unlike organic semiconductors, which typically show large Stokes-shifts (27). The nanosecond-transient absorption (TA) spectra of a $\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$ film is shown in Fig. 1B. We observed a sharp negative band peaking at 750 nm together with a broad positive band at shorter wavelengths, peaking around 550 nm. We assign the negative band to the photobleaching (PB) of the band gap or exciton transition, whereas the positive band represents a photoinduced absorption (PA). From biexponential data fitting, we retrieved the dominant time constant of both the PB and PA to be

~ 280 ns, followed by a long-lived tail (>1 μs). As we show in Fig. 1C, the PA band decay (288 ± 12 ns) mirrors the PB recovery dynamics (283 ± 6 ns), suggesting that the spectra arise from the same population. If we compare the transient absorption decays with the transient PL decay ($\tau_e = 273 \pm 7$ ns, where τ_e is the time taken for the PL to fall to $1/e$ of its initial intensity), there is a strikingly close match. This indicates that the decay of the radiative species we are monitoring with the PL represents the decay of all absorbing species (free carriers or weakly bound

excitons) in the perovskite film. We can therefore use PL-quenching measurements to determine a relevant diffusion constant and length in the perovskite films. We cannot, however, categorically determine whether this corresponds to the diffusion of free charge or excitons, but in either case, it will represent the relevant diffusion length for charge extraction in the solar cell. Additionally, we can safely exclude any contribution to the PL from trap states, which would be at lower energy and whose presence would otherwise complicate the subsequent PL-quenching analysis.

Fig. 2. PL measurements and fits to the diffusion model for the mixed halide and triiodide perovskites in the presence of quenchers. (A) Cross-sectional SEM image of a 270-nm-thick mixed halide absorber layer with a top hole-quenching layer of Spiro-OMeTAD. (B and C) Time-resolved PL measurements taken at the peak emission wavelength of the (B) mixed halide perovskite and (C) triiodide perovskite with an electron (PCBM; blue triangles) or hole (Spiro-OMeTAD; red circles) quencher layer, along with stretched exponential fits to the PMMA data (black squares) and fits to the quenching samples by using the diffusion model described in the text (details are available in the supplementary materials). A pulsed (0.3 to 10 MHz) excitation source at 507 nm with a fluence of 30 nJ/cm² impinging on the glass substrate side. (Inset) Comparison of the PL decay of the two perovskites (with PMMA) on a longer time scale, with lifetimes τ_e quoted as the time taken to reach $1/e$ of the initial intensity.

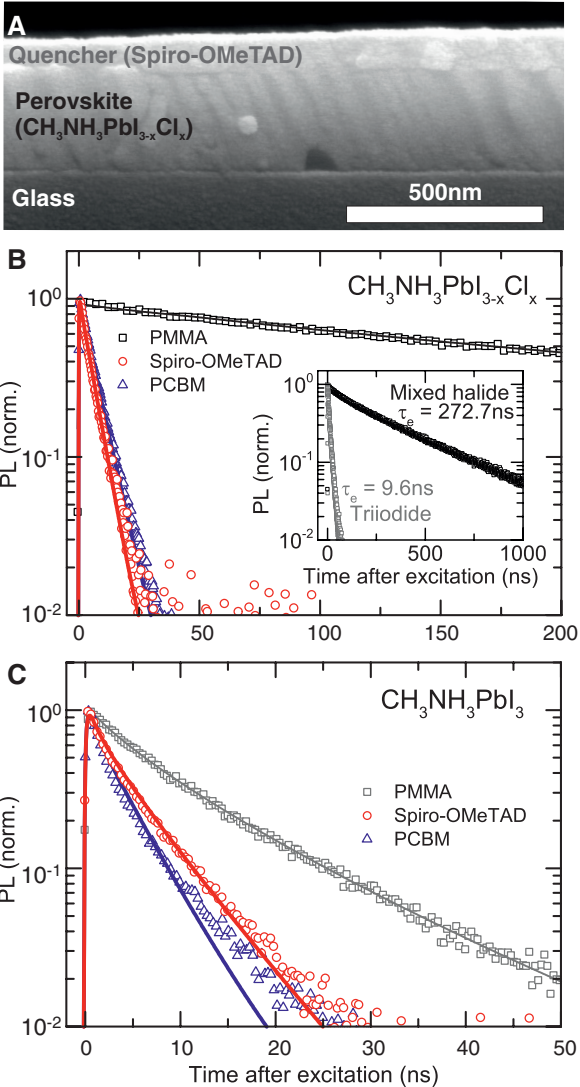


Table 1. Values for diffusion constants (D) and diffusion lengths (L_D) from fits to PL decays using the diffusion model described in the text. The errors quoted predominantly arise from perovskite film thickness variations, which are ± 35 nm for the triiodide perovskite films and ± 40 nm for the mixed halide perovskite films.

Perovskite	Species	D (cm ² s ⁻¹)	L_D (nm)
$\text{CH}_3\text{NH}_3\text{PbI}_{3-x}\text{Cl}_x$	Electrons	0.042 ± 0.016	1069 ± 204
	Holes	0.054 ± 0.022	1213 ± 243
$\text{CH}_3\text{NH}_3\text{PbI}_3$	Electrons	0.017 ± 0.011	129 ± 41
	Holes	0.011 ± 0.007	105 ± 32

The quenching samples were prepared by means of spin-coating layers of either a hole-acceptor (Spiro-OMeTAD) or an electron-accepting fullerene [phenyl-C₆₁-butyric acid methyl ester (PCBM)] on top of the perovskite films. In Fig. 2A, we show a scanning electron microscopy (SEM) image of a ~270-nm-thick CH₃NH₃PbI_{3-x}Cl_x absorber layer with a ~100-nm-thick Spiro-OMeTAD hole-quenching layer. We show SEM images of all sample configurations in figs. S1 and S2 and absorption spectra in fig. S3, which indicate that the mixed halide and triiodide perovskites have very similar bandgaps.

We present the time-resolved PL decays, measuring the peak emission at ~770 nm, for the mixed halide and triiodide perovskite absorbers in Fig. 2, B and C, respectively. The excitation fluence was kept to 0.03 μJ cm⁻²/pulse so as to ensure that nonlinear effects, such as exciton-charge annihilation, are unlikely to occur. The thickness of the CH₃NH₃PbI_{3-x}Cl_x films was 270 ± 40 nm, and the thickness of the CH₃NH₃PbI₃ films was 180 ± 35 nm, which is comparable with optimum device thicknesses. The corresponding steady-state spectra are shown in fig. S4. The PL decay of the neat CH₃NH₃PbI_{3-x}Cl_x film exhibits a time-constant of $\tau_e = 273 \pm 7$ ns. The addition of the PCBM and Spiro-OMeTAD electron and hole-quenching layers accelerates the PL decay, with observed time constants τ_e of 6.1 ± 0.1 ns and 5.1 ± 0.1 ns, respectively. In contrast, the lifetime for the neat CH₃NH₃PbI₃ film is only $\tau_e = 9.6 \pm 0.3$ ns, and this is quenched further, but not by such a large fraction, to 3.17 ± 0.03 ns for electrons and 4.2 ± 0.1 ns for holes.

The PL decay dynamics were modeled by calculating the number and distribution of excitations in the film $n(x,t)$ according to the one-dimensional diffusion equation

$$\frac{\partial n(x,t)}{\partial t} = D \frac{\partial^2 n(x,t)}{\partial x^2} - k(t)n(x,t) \quad (1)$$

where D is the diffusion coefficient and $k(t)$ is the PL decay rate in the absence of any quencher

material (further details are available in the supplementary materials) (22). The total decay rate k was determined by fitting a stretched exponential decay to the PL data measured from perovskite layers with PMMA. The effect of the quencher-layer was included by assuming that all photogenerated carriers that reach the interface are quenched, giving the boundary condition $n(L,t) = 0$, where $x = 0$ at the glass/perovskite interface and L is the perovskite film thickness. Because the samples were photo-excited from the glass substrate side of the samples, the initial distribution of photoexcitations was given by $n(x,0) = n_0 \exp(-\alpha x)$, where α is the absorption coefficient. The average diffusion length L_D of the species was then determined from $L_D = \sqrt{D\tau_e}$, where τ_e is the recombination lifetime in the absence of a quencher. If free charges are predominantly created upon photoexcitation, the PL decay represents the depopulation of charge carriers, and we estimated the diffusion coefficients for holes or electrons depending on which quenching layer is used. The results from the diffusion model fits are shown in Fig. 2, B and C, and the parameters summarized in Table 1. The diffusion lengths for both electrons and holes in the mixed halide perovskite are greater than 1 μm, which is much longer than the absorption depth of 100 to 200 nm. This indicates that there should be no requirement for meso- or nanostructure with this specific perovskite absorber. In contrast, the triiodide perovskite CH₃NH₃PbI₃ films have over one order of magnitude shorter diffusion length of ~100 nm for both electrons and holes, which is too short with respect to the absorption depth for this material to perform at the highest efficiencies in the thin-film configuration. The close similarity of the derived electron and hole diffusion coefficients and lengths in each of these perovskites either indicates very similar mobility for both electrons and holes, or it indicates that the predominant diffusion species is the weakly bound exciton. We can unfortunately not discriminate between the two from these results.

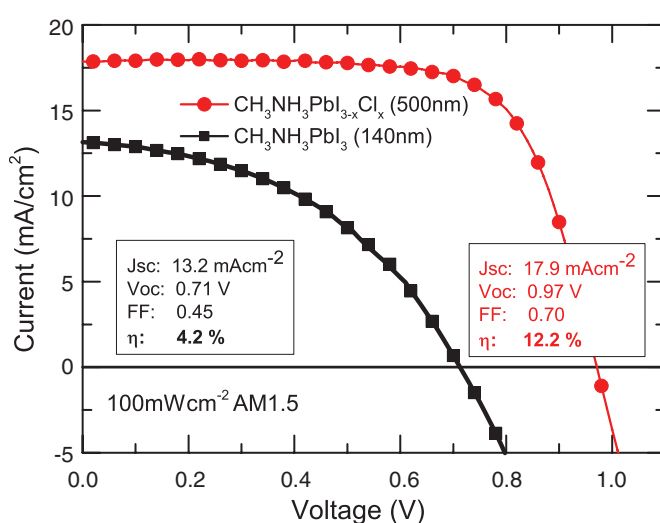
To confirm the importance of the determined diffusion length in full solar cells, and to test whether the measurement correlates well with device results, we fabricated solution-processed thin-film planar heterojunction solar cells with both the mixed halide and triiodide perovskites. We formed solid perovskite films on fluorine-doped tin oxide (FTO)-coated glass substrates, coated with an n-type TiO₂ compact layer so as to ensure selective collection of electrons at the FTO substrate. Subsequently, p-type spiro-OMeTAD was deposited as a hole-transporting layer so as to form a planar p-i-n heterojunction architecture. Current-voltage characteristics for the best devices fabricated are shown in Fig. 3. The CH₃NH₃PbI_{3-x}Cl_x planar heterojunction solar cells reach power conversion efficiencies in excess of 12%, which is the highest solution-processed planar heterojunction perovskite efficiency reported to date. In contrast, we were only able to attain η of ~4% with the CH₃NH₃PbI₃. Upon optimization of devices to obtain the highest efficiencies, we found that a thick layer of CH₃NH₃PbI_{3-x}Cl_x was preferable (~500 nm). However, the best CH₃NH₃PbI₃ cells had a perovskite layer thickness of only 140 nm. This observation is consistent with the diffusion-length calculations, in which the triiodide films are limited by the ~100-nm diffusion length, so that photogenerated charge in thicker films cannot be efficiently extracted before recombining. A full set of device results and average performances is given in table S1.

This work indicates that with correct tuning of the perovskite absorber, nano- or mesostructures are not necessary in order to achieve highly efficient charge generation and collection. Our results hence pave the way for further advances in planar heterojunction perovskite solar cells. Fundamentally, there still remain many open questions for the community concerning the nature of the excited state, the relative fraction of free and bound charge pairs at room temperature, and the interplay between the two species. Furthermore, this work introduces a new question as to why the small addition of chloride ions to the organolead triiodide perovskite results in such a striking increase in the electron-hole diffusion length, predominantly arising from a substantial inhibition of nonradiative electron-hole recombination. Understanding these subtleties will enable further improvement of the current family of materials.

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Fig. 3. Current-voltage curves for optimized planar heterojunction perovskite solar cells. CH₃NH₃PbI_{3-x}Cl_x (red line, circle symbols) and CH₃NH₃PbI₃ (black line, square symbols) cells were both measured under 100 mW cm⁻² AM1.5 simulated sunlight. J_{sc} is the short-circuit current, V_{oc} is the open-circuit voltage, FF is the fill factor, and η is the power conversion efficiency.



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Supplementary Materials

www.sciencemag.org/content/342/6156/341/suppl/DC1

Materials and Methods

Figs. S1 to S4

Tables S1 to S3

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Long-Range Balanced Electron- and Hole-Transport Lengths in Organic-Inorganic $\text{CH}_3\text{NH}_3\text{PbI}_3$

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Low-temperature solution-processed photovoltaics suffer from low efficiencies because of poor exciton or electron-hole diffusion lengths (typically about 10 nanometers). Recent reports of highly efficient $\text{CH}_3\text{NH}_3\text{PbI}_3$ -based solar cells in a broad range of configurations raise a compelling case for understanding the fundamental photophysical mechanisms in these materials. By applying femtosecond transient optical spectroscopy to bilayers that interface this perovskite with either selective-electron or selective-hole extraction materials, we have uncovered concrete evidence of balanced long-range electron-hole diffusion lengths of at least 100 nanometers in solution-processed $\text{CH}_3\text{NH}_3\text{PbI}_3$. The high photoconversion efficiencies of these systems stem from the comparable optical absorption length and charge-carrier diffusion lengths, transcending the traditional constraints of solution-processed semiconductors.

An ideal solar cell material should combine good optical absorption characteristics with efficient charge-transport properties. Low-temperature solution-processed light-harvesting films prepared by techniques such as spin-coating and chemical bath deposition are typically amorphous or poorly crystalline (1–3), consequently suffering from poor charge-carrier transport (4). This limitation necessitates device designs that decouple light absorption and charge-carrier transport lengths, including light-trapping strategies such as plasmonics (5, 6) as well as the

sensitized solar cell architecture (7, 8). The recent development of organic-inorganic halide perovskite materials such as $\text{CH}_3\text{NH}_3\text{PbI}_3$ as light harvesters in solid-state sensitized solar cells has led to reports of impressive efficiency values of up to 15% (9). This remarkable material has been used in a variety of photovoltaic architectures. A configuration used by Kim *et al.* (10) and Heo *et al.* (11) sandwiches the thin perovskite layer between a rough mesoporous TiO_2 photoanode and a hole-transporting layer such as 2,2',7,7'-tetrakis(*N,N*-di-*p*-methoxyphenylamino)-9,9'-spirobifluorene (Spiro-OMeTAD). Lee *et al.* (12) have shown that efficient solar cells can be fabricated by replacing the TiO_2 photoanode with an insulating Al_2O_3 scaffold, implying good electron-transport properties. Unexpectedly, Etgar *et al.* (13) reported an efficiency of 5.5% in a configuration without the hole-transporting layer, indicating good hole-transport properties. These indications of ambipolar charge-transport capabilities are supported by a recent report by Ball *et al.* (14) that demonstrated that ~350-nm-thick planar films sandwiched between a TiO_2 compact layer and a hole-transporting layer can generate short-circuit current densities of 15 mA/cm^2 . These reports together imply that the electron- and hole-transport

lengths within these organic-inorganic hybrid materials are high. Nonetheless, the innate dynamics of the photoexcited electrons and holes in $\text{CH}_3\text{NH}_3\text{PbI}_3$ driving the high efficiencies in these solar cells are unknown. Herein, through femtosecond transient optical spectroscopy of $\text{CH}_3\text{NH}_3\text{PbI}_3$ heterojunctions with selective electron and hole extraction, we successfully decoupled electron and hole dynamics and show evidence of long electron- and hole-transport lengths (both over 100 nm). Our findings indicate that this class of materials does not suffer from the bottleneck of low collection lengths that handicap typical low-temperature solution-processed photovoltaic materials.

In this study, electron-extraction layers [such as [6,6]-phenyl- C_{61} -butyric acid methyl ester (PCBM), C_{60}] with conduction band levels below that of $\text{CH}_3\text{NH}_3\text{PbI}_3$ and hole-extraction layers [such as Spiro-OMeTAD, poly(3,4-ethylenedioxythiophene) poly(styrenesulfonate) (PEDOT:PSS)] with valence band levels above $\text{CH}_3\text{NH}_3\text{PbI}_3$ were interfaced to $\text{CH}_3\text{NH}_3\text{PbI}_3$ to permit decoupling of the electron and hole dynamics (fig. S1). Comparing measurements on bare $\text{CH}_3\text{NH}_3\text{PbI}_3$ against $\text{CH}_3\text{NH}_3\text{PbI}_3$ /hole acceptor bilayers and $\text{CH}_3\text{NH}_3\text{PbI}_3$ /electron acceptor bilayers enables identification of electron and hole signatures in the organic-inorganic halide. Under identical experimental conditions, the photoluminescence (PL) quantum yield of the 65-nm-thick $\text{CH}_3\text{NH}_3\text{PbI}_3$ is greatly reduced when the perovskite is interfaced with an electron-extracting PCBM layer or a hole-extracting Spiro-OMeTAD layer (Fig. 1A). The PL intensity is quenched by a factor of 12.5 in the bilayer with Spiro-OMeTAD and by a factor of 50 in the bilayer with PCBM (table S1). Given that the current configurations are ideal layered systems (figs. S2 and S3), these high degrees of PL quenching, comparable to closely blended donor-acceptor system, are particularly revealing (15–19). With a linear absorption coefficient of $5.7 \times 10^4 \text{ cm}^{-1}$ at 600 nm (Fig. 2A and fig. S4), near-homogenous generation of the charge carriers in these 65-nm $\text{CH}_3\text{NH}_3\text{PbI}_3$ layers can be ensured (20). The PL quenching is expected to originate from the charge-carrier extraction across the interface (21–27). Efficient PL quenching suggests that the charge-carrier diffusion length inside the $\text{CH}_3\text{NH}_3\text{PbI}_3$

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layer is comparable to or longer than the layer thickness (65 nm). Correspondingly, the PL lifetimes were also substantially shortened when $\text{CH}_3\text{NH}_3\text{PbI}_3$ was interfaced with the PCBM or

Spiro-OMeTAD layer (Fig. 1B), with fitted lifetimes and SEMs of 4.5 ± 0.3 , 0.37 ± 0.02 , and 0.64 ± 0.03 ns for $\text{CH}_3\text{NH}_3\text{PbI}_3$, $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{PCBM}$, and $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$, respectively.

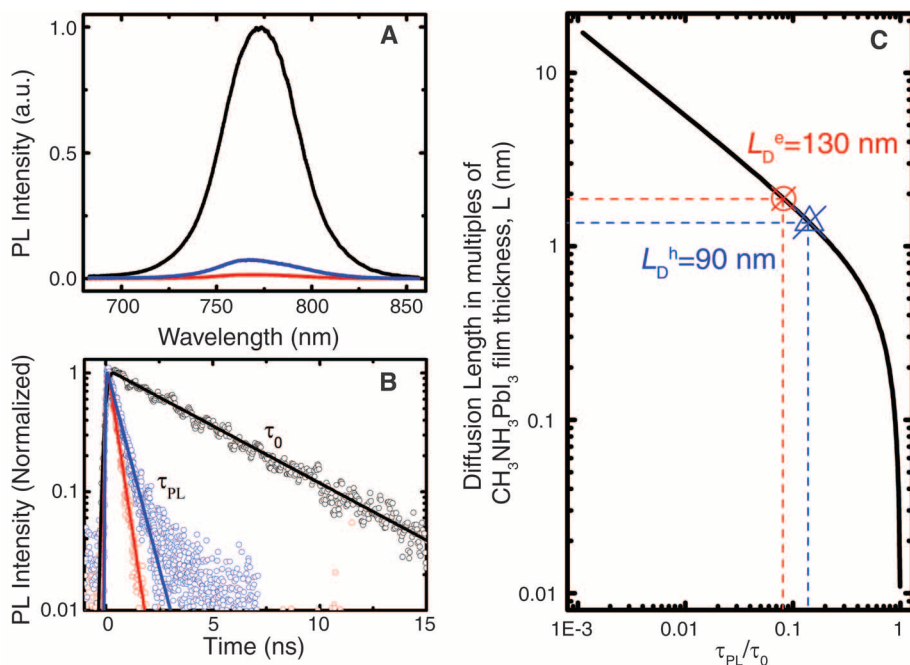


Fig. 1. Photoluminescence spectroscopy. (A) Time-integrated PL spectra and (B) Time-resolved PL decay transients measured at 760 ± 10 nm for quartz/ $\text{CH}_3\text{NH}_3\text{PbI}_3$ (65 nm) (black), quartz/ $\text{CH}_3\text{NH}_3\text{PbI}_3$ (65 nm)/PCBM (red), quartz/ $\text{CH}_3\text{NH}_3\text{PbI}_3$ (65 nm)/Spiro-OMeTAD (blue) films in vacuum after excitation at 600 nm (1 KHz, 150 fs, $1.3 \mu\text{J}/\text{cm}^2$). The solid lines in (B) are the single-exponential fits of the PL decay transients. a.u., arbitrary units. (C) A plot of exciton diffusion length versus PL lifetime quenching ratios based on Eq. S5. Diffusion length is scaled in multiples of $\text{CH}_3\text{NH}_3\text{PbI}_3$ layer thickness ($L = 65$ nm).

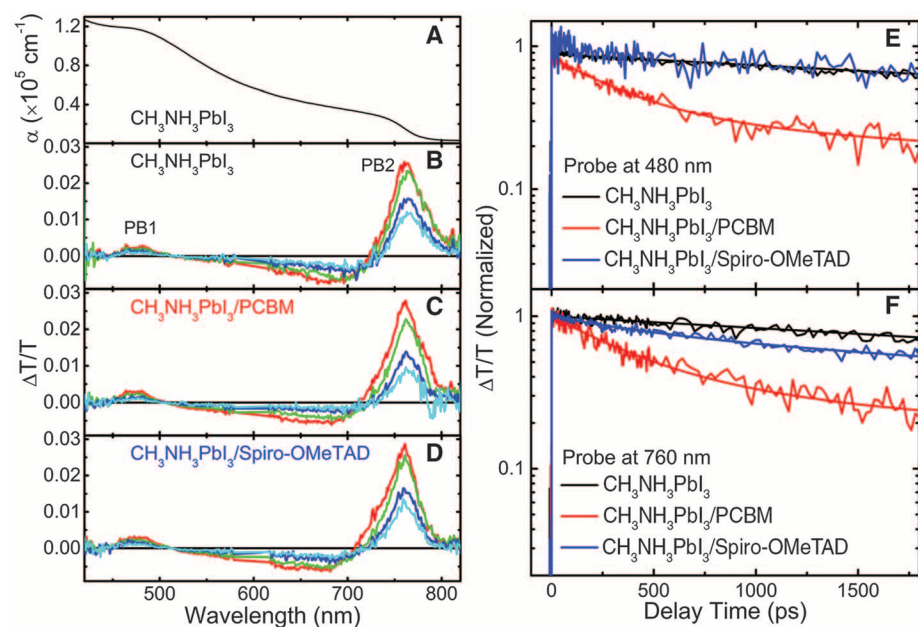


Fig. 2. Steady-state and transient absorption spectroscopy. (A) UV-visible absorbance spectrum for a pure $\text{CH}_3\text{NH}_3\text{PbI}_3$ layer. (B to D) Differential transmission ($\Delta T/T$) spectra for $\text{CH}_3\text{NH}_3\text{PbI}_3$, $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{PCBM}$, and $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$ films in vacuum after excitation at 600 nm (1 KHz, 150 fs, $1.3 \mu\text{J}/\text{cm}^2$): red (1 ps), green (100 ps), blue (500 ps), and cyan (1 ns). Normalized bleaching kinetics at (E) 480 and (F) 760 nm for the films in vacuum after excitation at 600 nm (1 KHz, 150 fs, $1.3 \mu\text{J}/\text{cm}^2$).

The single exponential PL decay indicates the good crystalline quality of the samples. By using the relation ($1/\tau_{\text{heterojunction}} = 1/\tau_{\text{perovskite}} + 1/\tau_{\text{CT}}$), the charge-carrier transfer time, τ_{CT} , and efficiency can be estimated to be 0.40 ns and 92%, respectively, for $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{PCBM}$ and 0.75 ns and 86% for $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$. The slight variation between the charge carrier transfer efficiencies obtained by using steady-state PL (Fig. 1A and table S1) and transient PL can be attributed to (i) extremely fast charge-carrier transfer at the interface (that cannot be monitored at the current temporal resolution) and (ii) the dependence of the steady-state PL on the light reflection, scattering, and refraction by the additional PCBM and Spiro-OMeTAD layers in the heterojunctions. Next, a charge-carrier extraction model based on diffusion was used to estimate the charge carrier diffusion lengths (see supplementary materials). Figure 1C shows the dependence of the charge-carrier diffusion length on the PL lifetime quenching ratios obtained from the analytical solution of the model. By assuming that charge-carrier quenching occurs only at the extraction layer interface with 100% efficiency, we obtained minimum estimates of the extracted electron and hole diffusion lengths of 130 and 90 nm. By comparison, solution-processed organic conjugated materials have typical diffusion lengths of about 10 nm (21–23), thermally deposited organic molecules have typical diffusion lengths of 10 to 50 nm (24–26), and colloidal quantum dot films have diffusion lengths of ~30 nm (organic cross-linked) and ~80 nm (hybrid surface passivated) (27). Thus, the conservatively estimated long diffusion lengths in the low-temperature solution-processed $\text{CH}_3\text{NH}_3\text{PbI}_3$ films compare favorably.

To improve the accuracy of these estimated values from the direct PL approach and to obtain more details on the photoexcited charge carrier dynamics, we also performed complementary transient absorption spectroscopy (TAS) measurements (10, 17, 28–33). Due to the large absorption coefficients and the long charge-carrier diffusion lengths, low pump fluence is essential to avoid extensive Auger recombination in $\text{CH}_3\text{NH}_3\text{PbI}_3$ (figs. S6 to S9). Figure 2A shows the linear absorption spectrum of $\text{CH}_3\text{NH}_3\text{PbI}_3$ spanning the ultraviolet (UV) to near infrared (800 nm) with two distinct peaks located at 480 and 760 nm, in agreement with earlier publications (9–14, 20). The second absorption peak (760 nm) is attributed to the direct gap transition from the first valence band maximum (VB1) to the conduction band minimum (CB1). However, the origin of the first absorption peak (480 nm) is still unresolved. Representative TA spectra of $\text{CH}_3\text{NH}_3\text{PbI}_3$ and its bilayer counterparts over the same spectral region are shown in Fig. 2, B to D, with two pronounced photobleaching (PB) bands. These long-lived PB peaks are located at almost the same spectral positions as the two absorption peaks. The bleaches at 480 and 760 nm are labeled as PB1 and PB2, respectively, and are attributed to

state-filling (34). For 600-nm photoexcitation, it is reasonable to attribute the 760-nm PB2 band to state-filling effects (which include the hole population of VB1, the electron population of CB1, and the interband stimulated emission) (10, 17, 28–33). However, it is not straightforward to assign the transitions associated with the 480-nm PB1 band. Given that the photoexcitation energy (of ~ 2.06 eV for 600-nm wavelength) is smaller than the energy of the PB1 peak (2.58 eV), only one of the two energy states involving this PB transition could be populated. The long-lived nature of this PB band further suggests that the populated energy level should be either VB1 or CB1 (see supplementary materials for a more detailed discussion of the assignment).

Upon selective excitation of the $\text{CH}_3\text{NH}_3\text{PbI}_3$ layer, no new PB or photoinduced absorption bands are observed when the electron or hole extraction layer is present. A comparative study at the respective probe wavelengths of PB1 and PB2 would thus yield detailed information about the charge-carrier dynamics. For pure $\text{CH}_3\text{NH}_3\text{PbI}_3$, the recombination dynamics at different probe wavelengths are relatively invariant over a range of pump fluences where second order effects are insignificant (fig. S6). All these decay transients are well fitted with a single exponential time constant of 5.6 ± 0.1 ns, which is longer than the measured PL lifetime of 4.5 ± 0.3 ns (table S1). Because time-resolved PL cannot monitor the recombination dynamics of all the photoexcited carriers, this finding suggests that the PL lifetime in pure $\text{CH}_3\text{NH}_3\text{PbI}_3$ is limited by the minority carrier lifetime. Correlating these PL lifetimes with the TA lifetimes of the bilayers allows us to identify the minority charge carriers.

With the PCBM (electron acceptor) layer present, both PB1 and PB2 bleaching peaks show an additional fast lifetime component of 0.37 ± 0.02 ns (Fig. 2, E and F), which is closely matched to the measured PL lifetime. This suggests that electrons are the minority charge carriers in $\text{CH}_3\text{NH}_3\text{PbI}_3$. Because PB1 and PB2 dynamics are simultaneously affected by the electron ex-

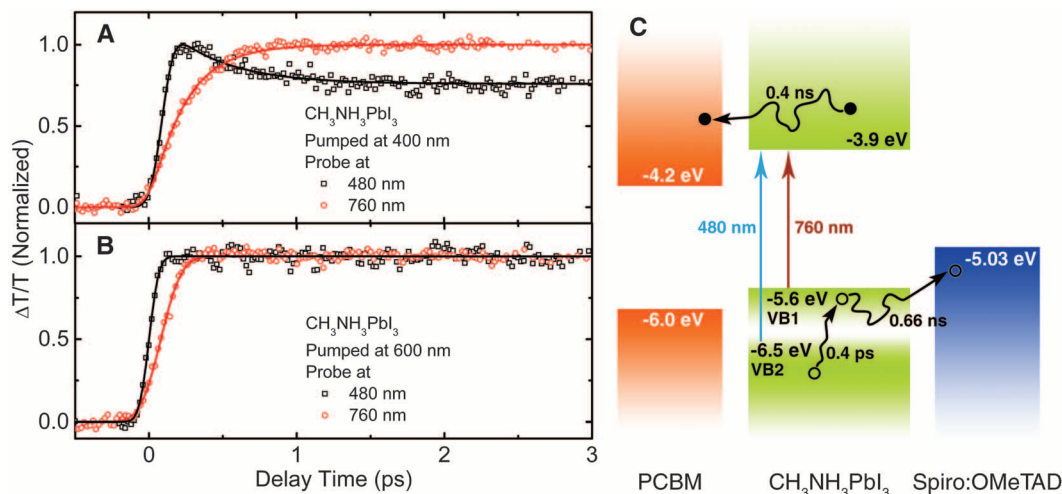
traction layer, the probes monitor the electron population in the CB1. For the $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$ (hole acceptor) samples, only PB2 exhibits an additional fast decay lifetime of 0.59 ± 0.03 ns (Fig. 2E), which is slightly faster than the PL lifetime of 0.64 ± 0.03 ns (table S1). This indicates that PB2 also reflects the hole population of VB1 (i.e., the transitions between VB1 and CB1). PB1 on the other hand is only related to the electron population in CB1 [i.e., the transitions between the lower valence band (VB2) and CB1] (Fig. 3C). By comparing the PB1 decays between pure $\text{CH}_3\text{NH}_3\text{PbI}_3$ and $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{PCBM}$, we determined the electron extraction time and efficiency in $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{PCBM}$ to be 0.40 ± 0.05 ns and 68%, respectively. Figure 2E also shows that about 27% of the photogenerated electrons are possibly trapped and therefore contribute neither to the electron extraction from $\text{CH}_3\text{NH}_3\text{PbI}_3$ to PCBM nor to the radiative recombination. By comparing the decay at PB2 between pure $\text{CH}_3\text{NH}_3\text{PbI}_3$ and $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$, we estimate the hole extraction time in $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$ to be 0.66 ± 0.05 ns. However, given that the TA signal at PB2 is a combination of signals from both electrons and holes, it is difficult to estimate the detailed hole-extraction efficiency at this stage.

The origins of PB1 and PB2 suggest the possibility of hot holes cooling from VB2 to VB1 after excitation of $\text{CH}_3\text{NH}_3\text{PbI}_3$ across the VB2-CB1 gap. Such hot-hole cooling dynamics could be validated through varying the pump wavelengths. After 3.10-eV (400-nm) excitation, Fig. 3A shows a very fast bleach buildup for PB1, which is close to the 150-fs laser pulse duration. Subsequently, hole localization from VB2 to VB1 occurs (within ~ 0.4 ps). The decay of the PB1 transient (indicative of the depopulation of VB2) is well matched with a concomitant rise of the bleach signal at PB2 (indicative of VB1 being populated), both at 0.4 ± 0.1 ps. On the other hand, excitations with lower energy photons [e.g., across the VB1-CB1 gap using 2.07-eV (600-nm) pulses] do not excite carriers in VB2, and therefore such hot-hole

cooling dynamics are absent (Fig. 3B). This 0.4-ps hot-hole cooling is much slower than that in most organic semiconductors (~ 100 fs) (30, 35). Potentially, these hot-hole energies could be efficiently extracted before the hot holes cool down to VB1 through optimizing the device configuration.

From fitting the TA decay dynamics with the diffusion model, we found the electron and hole diffusion coefficients to be 0.036 and 0.022 cm^2/s , respectively. By using these values, we calculated the electron and hole diffusion lengths (L_D) perpendicular to the film surface to be $L_D^e = 130$ nm and $L_D^h = 110$ nm, where $L_D = \sqrt{D\tau_{TA}}$. As expected, the L_D^h (majority carrier diffusion length) determined here is longer than that extracted from the more-direct PL approach presented earlier, which is sensitive to the minority carrier dynamics. The long transport lengths associated with $\text{CH}_3\text{NH}_3\text{PbI}_3$ are linked to its crystal structure, which consists of corner-connected PbI_6 octahedra that form a three-dimensional framework (36). Other organic-inorganic halide materials based on Sn have also displayed good charge-transport properties (37, 38). The slightly shorter diffusion length of the holes compared with the electrons is consistent with the hole's larger effective mass and larger positive space charge-limited transport. Nonetheless, these values are relatively balanced compared with typical values reported in bulk heterojunction solar cells, where the electron- and hole-transport lengths (proportional to their mobility) differ by orders of magnitude, resulting in space charge-limited photocurrents (39). These balanced long charge-carrier diffusion lengths would account for the remarkable performances reported for these $\text{CH}_3\text{NH}_3\text{PbI}_3$ devices. These L_D values are underestimated mainly because of the assumption that no quenching at the $\text{CH}_3\text{NH}_3\text{PbI}_3$ -quartz or -vacuum interfaces occur. The measured carrier lifetimes, τ_0 , are more susceptible to the non-ideality of these interfaces in these thinner spin-coated $\text{CH}_3\text{NH}_3\text{PbI}_3$ layers, leading to smaller τ_0 and consequently shorter L_D . Measurements in more "bulk-like" samples would yield longer

Fig. 3. Early time dynamics. Normalized bleaching kinetics at 480 and 760 nm in a short time range show the intervalence band hot-hole cooling for $\text{CH}_3\text{NH}_3\text{PbI}_3$ film (in vacuum) after excitation at (A) 400 nm ($1 \mu\text{J}/\text{cm}^2$) and (B) 600 nm ($1.3 \mu\text{J}/\text{cm}^2$). (C) A schematic illustrating the hot-hole cooling and charge recombination within $\text{CH}_3\text{NH}_3\text{PbI}_3$ and charge separation at the $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{PCBM}$ and $\text{CH}_3\text{NH}_3\text{PbI}_3/\text{Spiro-OMeTAD}$ interfaces. The approximate positions of VB1 and VB2 were obtained from the TA measurements.



τ_0 and higher L_D (submicrometer) (Fig. 1C). From the linear absorption coefficients (Fig. 2A), the absorption lengths are $L_\alpha \sim 100$ nm (at $\lambda = 500$ nm). These conservatively estimated carrier diffusion lengths measured in $\text{CH}_3\text{NH}_3\text{PbI}_3$ are comparable to the optical absorption lengths for $\lambda \leq 500$ nm but are shorter than the absorption lengths at longer wavelengths. Increasing the optical thickness of these layers through light-trapping architectures compensates for this slight mismatch, accounting for the high photoconversion efficiencies reported in these systems (9–14, 40).

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- Typically, PB peaks in TAS can arise from Coulomb interaction or state filling of the quasi-particles. In the former case, coulombic interaction among the excitons gives rise to a shift in energy of the probe beam–induced transitions, which occur in the vicinity of the excitons generated by the earlier pump beam. Such a phenomenon is commonly observed in quantum-confined low-dimensional systems or under high fluence excitation. In this mechanism, the occurrence of the PB peaks usually coincides with the occurrence of adjacent photoinduced absorption peaks because of the shift or broadening of the absorption peak. Furthermore, the PB peak positions will also shift with increasing pump fluence. The absence of photoinduced absorption peaks or pump fluence dependence of the PB peaks in these $\text{CH}_3\text{NH}_3\text{PbI}_3$ films allows us to rule out Coulomb interaction. On the other hand, state filling arises because of the changes in population of the various electronic states brought about by the initial pump beam. Hence, it will only influence probe beam–induced transitions that involve electronic states with changed populations.
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Supplementary Materials

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Materials and Methods

Fig. S1 to S9

Table S1

References (41, 42)

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Product-to-Parent Reversion of Trenbolone: Unrecognized Risks for Endocrine Disruption

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Trenbolone acetate (TBA) is a high-value steroidal growth promoter often administered to beef cattle, whose metabolites are potent endocrine-disrupting compounds. We performed laboratory and field phototransformation experiments to assess the fate of TBA metabolites and their photoproducts. Unexpectedly, we observed that the rapid photohydration of TBA metabolites is reversible under conditions representative of those in surface waters (pH 7, 25°C). This product-to-parent reversion mechanism results in diurnal cycling and substantial regeneration of TBA metabolites at rates that are strongly temperature- and pH-dependent. Photoproducts can also react to produce structural analogs of TBA metabolites. These reactions also occur in structurally similar steroids, including human pharmaceuticals, which suggests that predictive fate models and regulatory risk assessment paradigms must account for transformation products of high-risk environmental contaminants such as endocrine-disrupting steroids.

Humans discharge a multitude of bioactive organic contaminants into receiving waters that adversely affect aquatic organisms (1–3). Risk assessment approaches for regulating

these contaminants often are simplistic, typically assuming that if degradation occurs, the associated ecological risk greatly decreases. However, there is growing sentiment that some environ-

mental transformation reactions result in minimal mitigation of risk, forming products that retain bioactive moieties, exhibit greater toxicity, or affect different biological end points (4, 5).

The androgenic steroid trenbolone acetate [TBA; 17 β -(acetyloxy)estra-4,9,11-trien-3-one] is an anabolic growth promoter implanted in over 20 million cattle annually (6, 7), with annual revenue attributable to its use likely exceeding \$1 billion (8). Given the extensive use of TBA, its dominant metabolite [17 α -trenbolone (17 α -TBOH)] and other known metabolites

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[17 β -trenbolone (17 β -TBOH) and trenbolone (TBO)] can be widespread in agricultural environments (9, 10). 17 α -TBOH and 17 β -TBOH are potent endocrine-disrupting compounds, with concentrations as low as 10 to 30 ng/liter causing skewed sex ratios and reduced fecundity in fish (7, 11). Nevertheless, observations of TBA metabolite occurrence have not yet translated to concern, because they are believed to exhibit limited persistence in receiving waters. For example, manufacturer studies used for regulatory approvals specifically point to limited ecosystem risks of TBA metabolites due to rapid photodegradation (12).

Accordingly, we previously identified the structure and bioactivity of TBA metabolite photoproducts, showing that 17 β -TBOH undergoes rapid photohydration (13) to yield 12,17-dihydroxy-estra-5(10),9(11)-diene-3-one (or simply 12-hydroxy-17 β -TBOH) (14, 15). An analogous C12-hydroxylated photoproduct was identified for TBO (15), whereas 17 α -TBOH yields both major C12 [12-hydroxy-17 α -TBOH (15)] and minor C5 (5-hydroxy-17 α -TBOH) hydroxylated photoproducts (supplementary text and figs. S1 and S2). Because these photoproduct mixtures retained bioactivity (15), we focused on photoproduct stability across a range of conditions representative of light (day) and dark (night) surface waters. When photoproduct transformation was observed, we used liquid chromatography high-resolution tandem mass spectroscopy (LC-HRMS/MS) and nuclear magnetic resonance (NMR) to characterize the resulting product structures and the potential for persistent bioactivity in surface waters (16).

Simulated day-night cycling experiments conducted over 72 hours at pH 7, 25°C revealed unexpected persistence of TBA metabolites (Fig. 1A). For example, 17 α -TBOH decay during irradiation was consistently followed by concentration rebound during subsequent dark periods, with equivalent rates of photodecay and dark regeneration for each diurnal cycle. Both NMR (figs. S3 to S10) and LC-HRMS/MS confirmed that 17 α -TBOH was regenerated in the dark. This dynamic behavior appears to be linked to the thermal (i.e., nonphotochemical) instability of C5 and C12 hydroxylated photoproducts, which decayed concurrently with 17 α -TBOH regrowth.

A noteworthy feature of both 5- and 12-hydroxy-17 α -TBOH is their allylic alcohol moiety (Fig. 1A). Steroidal allylic alcohols are prone to dehydration but typically under more aggressive conditions (e.g., low pH and high temperature) (17). However, our data suggest that 5- and 12-hydroxy-17 α -TBOH dehydrate under ambient conditions, with the driving force being regeneration of the conjugated trienone system. This coupled photohydration-dehydration mechanism results in net reversibility of 17 α -TBOH photolysis, a product-to-parent reversion mechanism also observed for other TBA metabolites (Fig. 1B). 17 β -TBOH and TBO exhibited reduced regrowth during 12-hour dark periods (~1% of initial mass) relative to 17 α -TBOH (~15%), which

we attribute to their photoproducts being more susceptible to concurrent phototransformation and hydroxylation rather than dehydration (supplementary text and fig. S11).

We also simulated TBA metabolite transport from the photic zone of surface water into darker regions (such as lake hypolimnia, hyporheic zones, or benthic sediments) by photolyzing TBA metabolite solutions to >99% transformation (pH 7, 6 hours irradiation) and then storing the resulting photoproduct mixtures in the dark for 5 days at 25°C. Consistent with the diurnal cycling experiments, 17 α -TBOH exhibited substantial reversion, with over 60% of its initial mass recovered after 120 hours, whereas 17 β -TBOH and TBO yielded 10% recovery over this period (Fig. 2A).

The rates and extents of product-to-parent reversion were highly dependent on solution conditions, with some promoting rapid and near-complete TBA metabolite regeneration. For example, $88 \pm 3\%$ of the initial 17 α -TBOH mass was regenerated nearly instantaneously when photoproduct mixtures were acidified to pH 2, with slightly lower recoveries ($66 \pm 5\%$) when raised to pH 12 (Fig. 2A). Reversion of 17 β -TBOH and TBO was also acid-catalyzed ($65 \pm 7\%$ and $32 \pm 4\%$ recovery, respectively), but their regrowth was more lim-

ited at pH 12. We believe that these recoveries at pH 2 probably reflect the maximum photoproduct mass available for reversion.

The reversion of 17 α -TBOH was also enhanced at pH 9 and pH 5, at least initially, relative to pH 7 (Fig. 2B). Thus, acid- and base-catalyzed reversion will probably result in higher 17 α -TBOH concentrations in mildly acidic or alkaline waters. In fact, the initial rate of reversion at pH 5 is fast enough to slow 17 α -TBOH phototransformation relative to the rate observed at neutral pH (supplementary text and fig. S12). In manufacturer regulatory studies, slower rates of 17 α -TBOH phototransformation at pH 5 relative to pH 7 were attributed, we believe incorrectly, to variations in solar irradiance from cloudy weather experienced during data collection at pH 5 (12).

Reversion is also temperature-dependent. At pH 7, rates of 17 α -TBOH regrowth increased nearly 30-fold from 5° to 35°C (Fig. 2C), a temperature range representative of seasonal variations. Faster reversion rates at 35°C ultimately allowed more complete 17 α -TBOH recovery in the dark (30% over 12 hours and 75% over 60 hours). Accordingly, we expect photoproduct-to-parent reversion to be most prominent in warm sunlit waters, whereas colder winterlike conditions

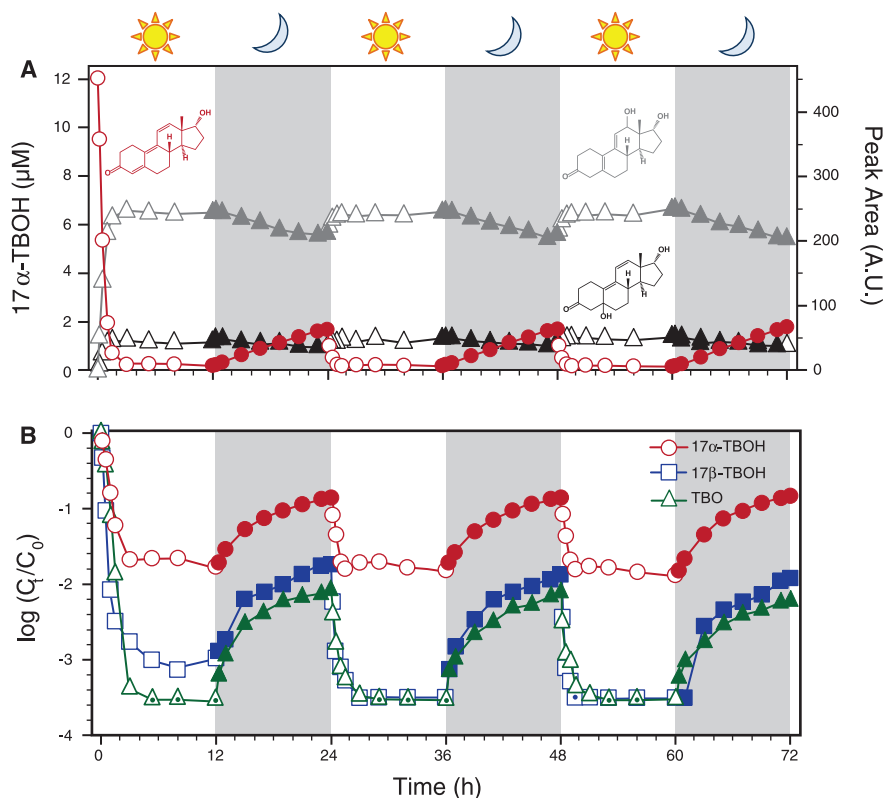


Fig. 1. Diurnal cycling of TBA metabolites. (A) Day-night cycling of 17 α -TBOH (red), 12-hydroxy-17 α -TBOH (gray), and 5-hydroxy-17 α -TBOH (black) at pH 7, 25°C. Data for 17 α -TBOH are presented as aqueous concentration (left axis), whereas peak areas from spectrophotometric absorbance [wavelength (λ) = 350 nm for 17 α -TBOH; λ = 254 nm for photoproducts] are also presented (right axis). (B) Corresponding data for 17 β -TBOH and TBO, where symbols with a center dot indicate values below our detection limit. In these cases, data points correspond to this limit (~3 nM) for 17 β -TBOH and TBO. C/C_0 , time-dependent concentration normalized to initial concentration; h, hours; A.U., arbitrary units.

Fig. 2. Dark regrowth of TBA metabolites and the influence of pH and temperature on reversion. (A) Regeneration of 17 α -TBOH, 17 β -TBOH, and TBO in photoproduct mixtures stored in the dark (pH 7, 25°C). hv, light energy. The inset reports the percent recovery (mean and standard deviation of triplicate analyses) when photoproduct mixtures were adjusted to pH 2 or pH 12. Also shown is the regeneration of 17 α -TBOH as a function of (B) pH (5, 7, and 9 at 25°C) and (C) temperature (5, 15, 25, and 35°C at pH 7) from photoproduct mixtures. In (B), the initial rate of regrowth obtained from linear regression analysis was enhanced at pH 9 (0.3 μ M hour⁻¹) and, at least initially [time (*t*) < 2 hours], at pH 5 (0.6 μ M hour⁻¹) relative to pH 7 (0.17 μ M hour⁻¹). The slower rate observed over longer time scales at pH 5 (0.07 μ M hour⁻¹) probably reflects differences in the dehydration rates of 5- and 12-hydroxy-17 α -TBOH.

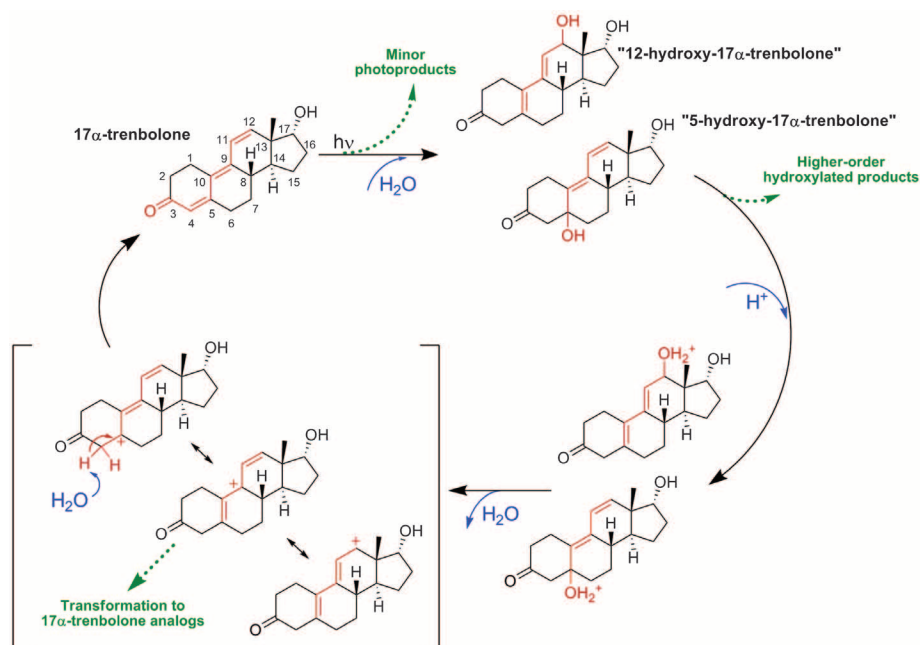
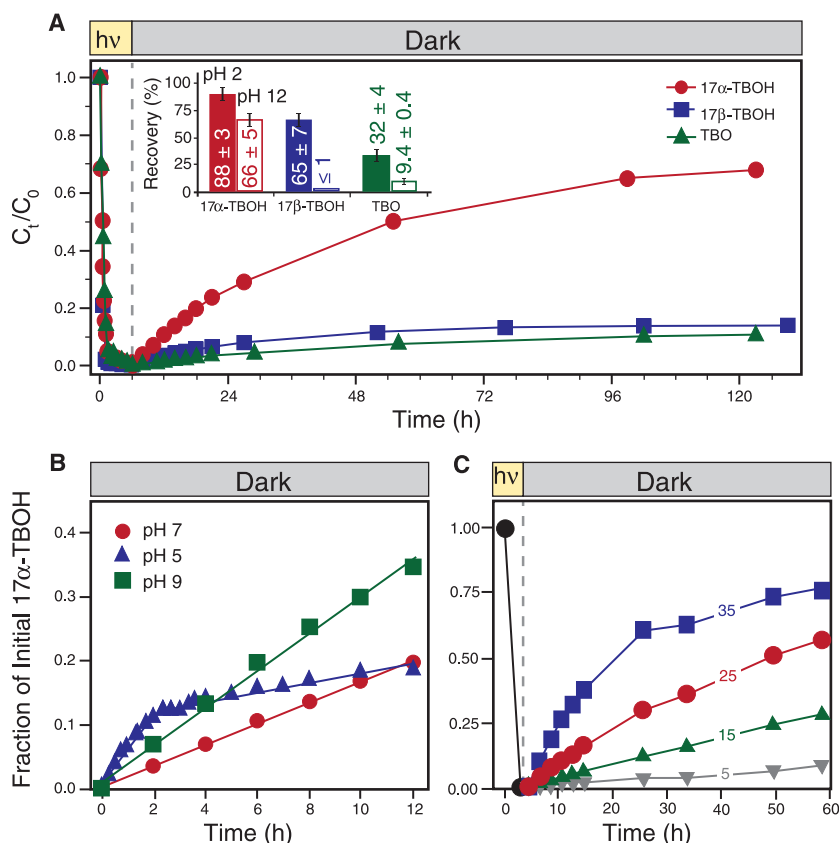


Fig. 3. Proposed acid-catalyzed reversible photohydration of 17 α -TBOH. Dashed green arrows indicate pathways that break the product-to-parent reversion cycle. Red highlights detail the changes in the trienone structure during photolysis and subsequent dehydration.

should promote photoproduct stability by slowing dehydration.

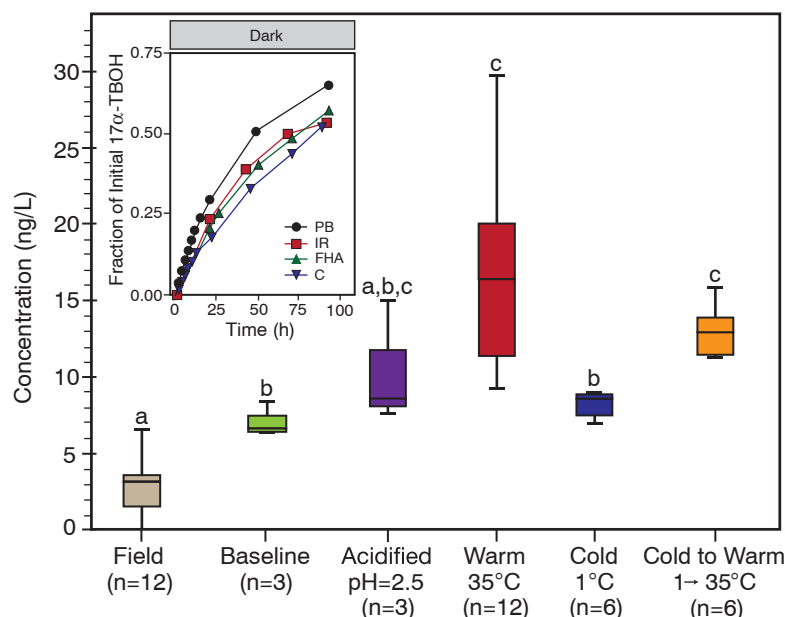
Accounting for these effects, we can now present a more complete characterization of TBA metabolite fate in surface waters. Consistent with established

pathways for allylic sterols (17), we propose that dehydration at moderately acidic to low pH proceeds via unimolecular (E1) elimination involving the formation of a resonance-stabilized carbocation (Fig. 3). Parent regeneration from this carbocation

intermediate may be accompanied by concurrent transformations, including structural rearrangements and/or reaction with naturally abundant nucleophiles, to yield complex mixtures of isomers, structural analogs, and substituted derivatives of TBA metabolites with uncharacterized bioactivity. For example, while monitoring TBO photoproduct reversion at pH 5, we observed evidence of photoproduct interconversion and formation of a presumed structural analog that coelutes with TBO (supplementary text and figs. S13 to S18). These phenomena were not observed at neutral or basic pH, which is consistent with the proposed acid-promoted carbocation intermediate. At neutral and basic pH, dehydration is more likely to proceed via enol or enolate formation in parallel to reactions yielding higher-order hydroxylated products.

Beyond model laboratory systems, we have also observed reversion in more complex aquatic matrices, including Iowa River water (Fig. 4), as well as in experiments using environmentally relevant TBA metabolite concentrations (for example, ~420 ng/liter 17 β -TBOH; fig. S19). To explore reversion in agricultural receiving waters with competing attenuation pathways (such as sorption and biodegradation), we dosed a small collection pond on a rangeland with manure from TBA-implemented cattle (supplementary text). After overnight leaching increased 17 α -TBOH concentrations in the pond to 6.1 ng/liter, concentrations decreased by ~50% during the day, consistent with phototransformation. At sunset, a large (31-liter) sample was collected and processed so that

Fig. 4. 17 α -TBOH dynamics in a field mesocosm and complex aquatic matrices. After dosing the collection pond (at 22:00), field samples ($n = 4$, in triplicate) were analyzed over 17 hours. At sunset (15:00), the baseline sample was processed and split into subsamples, which were either acidified to pH 2.5, incubated [warm, temperature (T) = 35°C], refrigerated (cold, $T = 1^\circ\text{C}$), or refrigerated then incubated after 24 hours (cold to warm, $T = 1^\circ \rightarrow 35^\circ\text{C}$). Group means are different [one-way analysis of variance: $F(5,37) = 17.5$, $P < 0.001$] if groups do not share the same letter as other groups (Games-Howell post-hoc test: $P < 0.05$). Lines, boxes, and whiskers represent median, interquartile, and minimum and maximum values, respectively. The inset shows regeneration of 17 α -TBOH in photoproduct mixtures (dark, 25°C) in solutions of 10 mg/liter Fluka Humic Acid (FHA), 250 mg/liter of bicarbonate as CaCO_3 (C), and Iowa River water (IR; turbidity, 15.1 nephelometric turbidity units; alkalinity, 196 mg/liter as CaCO_3 ; total hardness, 270 mg/liter as CaCO_3 ; total dissolved organic carbon, 16.6 mg/liter; pH 8.2.). Provided for comparison are data from a phosphate buffer (PB) system. Unless noted, all systems were at pH 7. The rate of reversion is less in Iowa River water than would be expected for its alkaline pH, probably because of the slight inhibition observed in model systems with Fluka Humic Acid and bicarbonate.



subsamples of this baseline sample could be subjected to different storage conditions (Fig. 4). Laboratory storage at 35°C significantly increased 17 α -TBOH concentrations (from 7 to 20 ng/liter; Games-Howell post-hoc test, $P = 0.003$), whereas samples stored at 1°C were statistically identical to the baseline sample (Games-Howell post-hoc test, $P = 0.65$). However, when the temperature of these 1°C samples was subsequently raised after 24 hours to 35°C, 17 α -TBOH concentrations statistically increased to 15 ng/liter (Games-Howell post-hoc test, $P = 0.005$). These data are consistent with expected trends for product-to-parent reversion dynamics under actual field conditions. Incidentally, we also observed similar decay and regrowth characteristics for a major uncharacterized, nontarget compound, probably steroidal, in our gas chromatography–tandem mass spectrometry chromatograms, whose occurrence was highly correlated to known TBA metabolites (figs. S20 to S22).

Although growth-promoting steroids provide indisputable economic and environmental advantages (such as reduced land use, nutrient loads, and greenhouse gas emissions) for animal agriculture (18), their use should not compromise environmental health. Contrary to prevailing assumptions of limited environmental persistence, product-to-parent reversion results in the enhanced persistence of TBA metabolites via a dynamic exposure regime that defies current fate models and ecotoxicology study designs. Reversion also provides a route to novel steroidal isomers, structural analogs, and derivatives, or “environmental designer steroids,” with as-yet-uncharacterized properties and risks. Temperature- and pH-dependent reversion rates also imply substantial uncertainty for nearly all existing TBA metabolite occurrence data. Collectively, these possibilities suggest the unrecognized occurrence of TBA metabolites and/or structurally similar, bioactive transforma-

tion products that may contribute to otherwise unexplained observations of endocrine disruption in agriculturally affected receiving waters (19, 20). For example, although chemical analyses often cannot identify causative agents, a recent study indicated widespread (35% of tested waters) androgenic activity in U.S. waters (21).

We also recently observed photoproduct-to-parent reversion for dienogest, a potent progestin used as an oral contraceptive (22), and for dienedione, an illicit anabolic steroid marketed as a “bodybuilding supplement” (fig. S23). Therefore, it appears that other pharmaceutical steroids, namely those with dienone and trienone moieties, also may exhibit enhanced environmental persistence and pose greater ecological risks than currently realized. In fact, although this work focused solely on phototransformation, we believe that the propensity for reversion is more generally linked to the conjugated enone moiety of TBA metabolites and these other pharmaceuticals. Ultimately, such observations of product-to-parent reversion illustrate that comprehensive assessment of environmental transformation products should be prioritized for high-risk contaminants such as endocrine-disrupting steroids. Further, the use of TBA or similar steroids that are equally prone to environmental transformations that preserve bioactive moieties may need to be reevaluated in favor of more-sustainable pharmaceutical technologies designed to ensure ecosystem protection.

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Supplementary Materials

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Materials and Methods

Supplementary Text
Figs. S1 to S23
Tables S1 to S15
References (23–29)

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Direct Spectroscopic Characterization of a Transitory Dirhodium Donor-Acceptor Carbene Complex

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A multitude of organic transformations catalyzed by dirhodium(II) (Rh₂) complexes are thought to proceed via the intermediacy of highly reactive, electrophilic carbenoid intermediates that have eluded direct observation. Herein, we report the generation of a metastable Rh₂-carbenoid intermediate supported by a donor-acceptor carbene fragment. This intermediate is stable for a period of ~20 hours in chloroform solution at 0°C, allowing for an exploration of its physical and chemical properties. The Rh=C bond, characterized by vibrational and nuclear magnetic resonance spectroscopy, extended x-ray absorption fine structure analysis, and quantum-chemical calculations, has weak σ and π components. This intermediate performs stoichiometric cyclopropanation and C–H functionalization reactions to give products that are identical to those obtained from analogous Rh₂ catalysis.

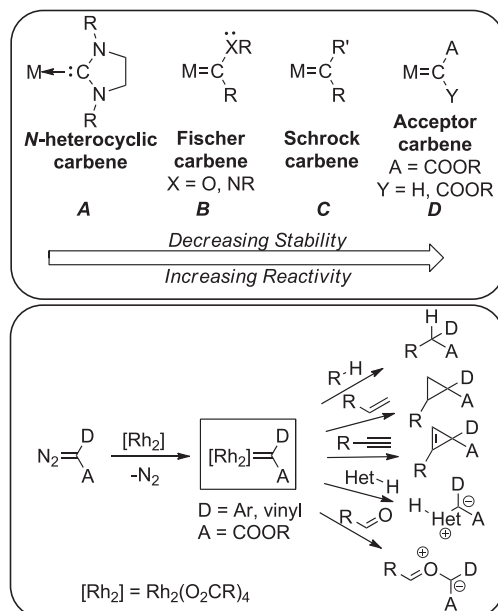
Isolation of reactive intermediates has historically provided a great deal of insight into the mechanisms of chemical reactions (1), and great progress has been made in the synthesis of stable analogs of reactive electron-deficient species such as carbenes and nitrenes (2). Carbenoids, or metal-carbene species, first postulated in 1952 (3), vary widely in their stability and reactivity. *N*-heterocyclic carbenes (A in Fig. 1), stabilized by two N atoms that flank the carbene C atom, are stable even in the absence of a metal but serve well as unreactive spectator ligands to metals (4, 5). When only one heteroatom is present, the carbene ligand is unstable, but the resulting metal complexes known as Fischer-type carbenes (B in Fig. 1) can be isolated and employed as stoichiometric reagents in reactions such as cyclopropanation (6). Schrock-type carbene complexes (C in Fig. 1) have no stabilizing heteroatoms on the carbene carbon and promote catalytic olefin metathesis (7). Addition of one or more electron-accepting groups (such as esters) to the carbene center destabilizes it further such that even the metal complexes are often too unstable to isolate (D in Fig. 1). Acceptor carbene complexes

are thus often proposed as key intermediates in a broad range of organic reactions. Outstanding among these are Rh₂ tetracarboxylate-catalyzed reactions of donor-acceptor diazocarbonyl compounds, which result in a wide range of synthetically useful transformations (8–18) (for example, Fig. 1, bottom). Additionally, the initial products of these reactions are often themselves highly reactive and can engage in domino sequences that lead to the rapid construction of complex

products (17). These systems are catalytically extremely efficient, capable of achieving turnover numbers in excess of 1,000,000 at rates of up to 300 turnovers per second (14).

Although some examples of mononuclear metal complexes of acceptor carbenes have been isolated (19–22), the Rh₂ carbenoid that is featured so prominently in the most efficient and synthetically useful transformations described above has long defied characterization. Our limited understanding of this intermediate has therefore relied on models that rationalize product distributions, computational studies, and limited kinetic studies (12, 23). This type of carbenoid intermediate has remained elusive because, in most instances, its formation is the rate-determining step in the catalytic cycle (23). The only known example of an isolated Rh₂ carbene complex is that of the stable nucleophilic Arduengo carbene (24), which does not engage in the above-mentioned electrophilic reactivity.

To observe an Rh₂-carbenoid intermediate, we have turned to donor-acceptor diazo esters, in which the donor group is typically aryl or vinyl (11, 25, 26). Both chemical and computational studies indicate attenuated reactivities for donor-acceptor carbenoids compared with acceptor-only carbenoids, so much so that the C–H functionalization step is predicted to have a similar activation energy to the carbenoid-generation step (26). This attenuation not only leads to highly selective transformations and very high catalyst



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turnover numbers, but it also implies substantial stabilization of the donor-acceptor carbenoid intermediate, suggesting that this intermediate may have a chance of being observed.

To quickly screen for a metastable carbenoid intermediate, a number of Rh_2 -carboxylate catalysts were subjected to a 500-fold excess of methyl 2-diazo-2-(4-methoxyphenyl)acetate (**2**) at 0°C (method A). These conditions allow for the generation of a steady-state concentration of the carbenoid intermediate, detectable by ultraviolet-visible (UV-Vis) spectroscopy. When $\text{Rh}_2(\text{tpa})_4 \cdot 2\text{CH}_2\text{Cl}_2$ (**1**, Fig. 2A, fig. S1; tpa = triphenylacetate) was used in this test, a new 700-nm UV-Vis feature attributed to carbenoid intermediate **3** was observed, having a half-life of ~10 s under ambient conditions (Fig. 2C). A second stoichiometric method to form a metastable solution of **3** was subsequently discovered (method B). In method B, rigorously dry, deoxygenated dichloromethane or chloroform solutions of **1** and **2** were combined in a 1/0.5 stoichiometry, respectively, producing an immediate effervescence. Methods A and B are summarized in Eq. 1 in Fig. 2; formation of **3** under both conditions is confirmed by similarity of the UV-Vis and Raman features of the intermediates formed by both methods (Fig. 2C and fig. S2). Intermediate **3** can also be formed under conditions amenable to observation by mass spectrometry. When a mixture of solids **1** and **2** is exposed to a matrix-assisted laser desorption/ionization mass spectrometry source, a signal at mass/charge ratio = 1532/1533 that corresponds to ^{12}C **3**/ ^{13}C **3** is observed (see Eq. 1 in Fig. 2 for the site of ^{13}C labeling). Carbene complex **3** is readily distinguished from light green precursor **1** by its intensely dark blue-green color. Solutions of **3** prepared by method B are stable under inert atmosphere at 0°C for up to 20 hours in chloroform or dichloromethane.

The intense absorption features of **3** allow for characterization of this intermediate by resonance Raman (rR) spectroscopy. Excitation of **3**, prepared by either method A or B and immediately frozen at -196°C, yields three vibrational stretches that display substantial $^{12}\text{C}/^{13}\text{C}$ isotope shifts (Fig. 2, D and E). These vibrations match well with computationally predicted Rh-C stretching and O-Rh-C bending modes, all due to an Rh=C interaction (Table 1 and fig. S3). These data are comparable to those reported for group VI Fischer carbene complexes, which exhibit carbene-based vibrational modes in the 700- to 900- cm^{-1} range. (27)

The ^1H nuclear magnetic resonance (NMR) spectrum (fig. S4) of a CDCl_3 solution of **3** (method B) clearly shows the presence of unreacted **1** [multiplets at chemical shift δ = 6.625, 6.87, and 7.08 parts per million (ppm)], as well as a new species (**3**) that has slightly lower-frequency aryl proton resonances (δ = 6.575, 6.82, and 7.04 ppm) in addition to signals due to the methoxy groups of the carbene fragment at δ = 2.75 ppm (ester) and 3.92 ppm (*p*-OMe; Me, methyl). The ester methoxy signal is drastically

shifted from its original position in **2** (3.85 ppm) due to the proximity of the tpa ligands to the aryl rings, as established by the observation of the

nuclear Overhauser effect between the methyl peak at δ = 2.75 ppm and the aryl resonances of **3** at δ = 6.575 and 6.82 ppm (fig. S5).

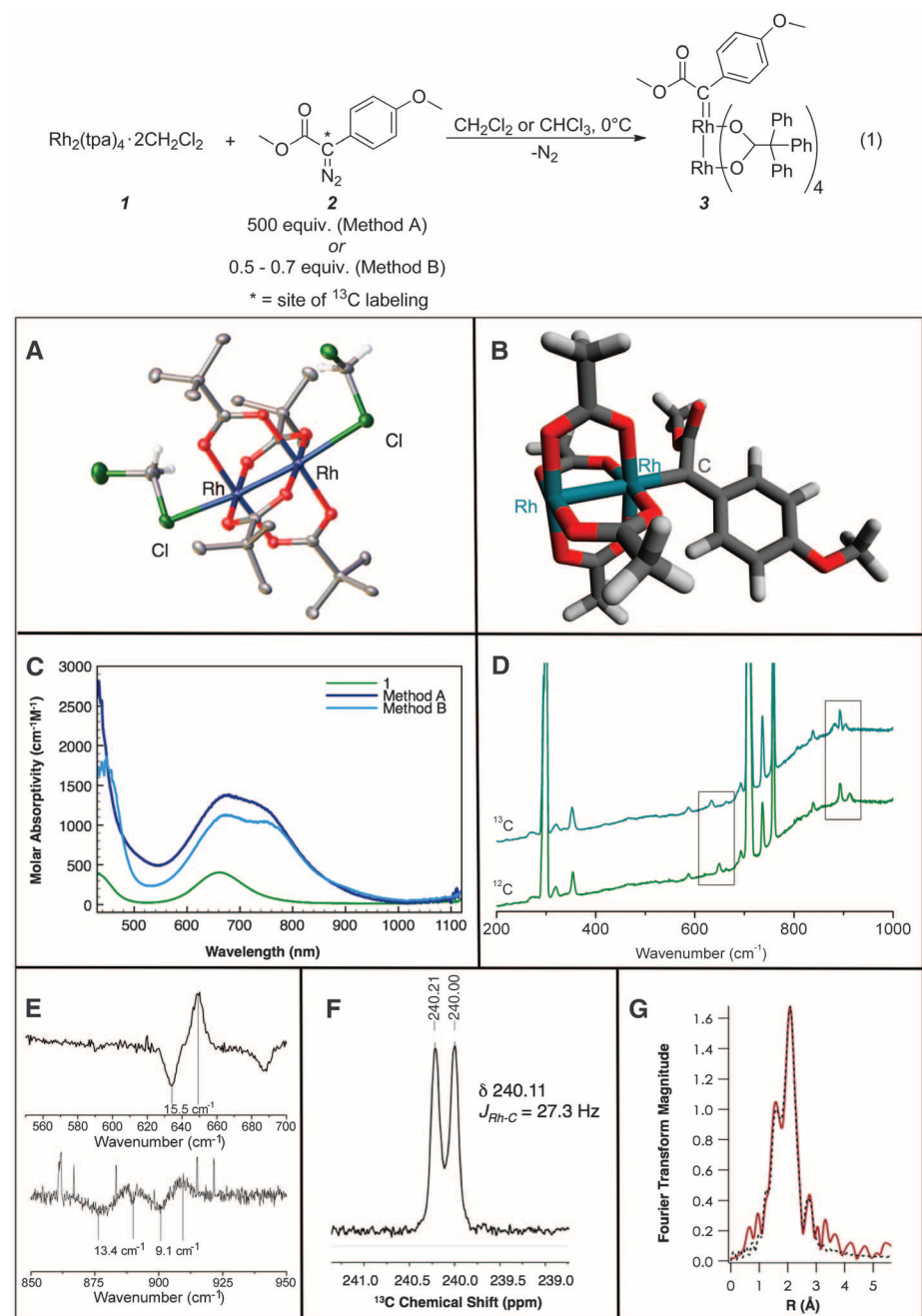


Fig. 2. Characterization of dirhodium carbene complex. (Top) Equation 1. Ph, phenyl. (Below) Characterization of dirhodium carbenoid **3**. (A) X-ray crystal structure of **1** with axially bound dichloromethane molecules. Only the *ipso*-carbon of each phenyl group of the tpa ligands is shown for clarity; thermal ellipsoids are each drawn at a 50% probability level. Red, oxygen; green, chlorine; blue, rhodium; gray, carbon; white, hydrogen. (B) Computational model of **3a** [B3LYP/Rh-RSC+4f, 6-31G(d)]. (C) UV-Vis spectra of **3** in chloroform at -78°C generated by method A (dark blue line) and method B (light blue); the spectrum of starting complex **1** (room temperature) is denoted by a green line. Molar absorptivity is given relative to the initial concentration of **1**. (D) Resonance Raman spectra of **3** and ^{13}C -labeled **3**. The boxes indicate regions where the isotopic shifts are observed. (E) Difference spectra of the boxed regions from (D) of the rR spectrum indicating the isotopic shifts. (F) Carbene resonance in the ^{13}C NMR trace of a 0°C solution of **3** in CDCl_3 (full spectrum shown in fig. S7). (G) Rh K-edge EXAFS for **3**. Experimental data are shown by the red line; the theoretical fit is denoted by the dashed gray line.

Using ^{13}C -labeled **2**, preparation of **3** allowed for observation of the highly deshielded carbenoid carbon by ^{13}C NMR at 242 ppm (Fig. 2F and fig. S7). This signal with $\delta > 200$ ppm is the hallmark of a transition metal carbene complex (28). Moreover, this signal is conspicuously split into a doublet (coupling constant $J_{\text{Rh-C}} = 27.3$ Hz), indicating a degree of covalent bonding between this C atom and a single ^{103}Rh nucleus (100% natural abundance with nuclear spin $I = 1/2$). In comparison, the previously reported Rh_2 Arduengo carbene complex (**24**) exhibits a carbene carbon resonance at 153.73 ppm, which is indicative

of a lesser degree of electrophilicity, consistent with its lack of carbene-transfer reactivity. Furthermore, the increased $J_{\text{Rh-C}}$ of 41.5 Hz in the Arduengo carbene compared with **3** implies a stronger $\text{Rh}=\text{C}$ interaction in the former. A mononuclear Rh phenyl carbene complex reported by Cohen *et al.* also displays an increased $J_{\text{Rh-C}}$ at 38.3 Hz and attenuated electrophilicity with a ^{13}C chemical shift at 173 ppm (29).

To obtain structural information for **3**, we measured Rh K-edge x-ray absorption spectroscopy, including extended x-ray absorption fine structure (EXAFS) for **1**, **3**, and a solution of **3**

decomposed by exposure to air (**3-dec**). Samples of **1**, **3**, and **3-dec** show no difference in their Rh K-edge energy, implying that intermediate **3** is best formulated as an $\text{Rh}_2(\text{II,II})$ species (fig. S8). EXAFS-derived bond distances for **1** show excellent agreement with corresponding distances determined for **1** by x-ray crystallography (table S1), lending confidence to the application of EXAFS to **3**.

The EXAFS data for **3** (fig. S9) reveal two important changes from the structure of **1**. First, the Rh-Rh bond distance in **3** elongates to 2.43 Å from 2.38 Å in **1**. Second, whereas the average number of light-atom (C or O) scatterers per Rh atom in **1** is four, the EXAFS data for **3** indicate 4.5 C or O scatterers bound per Rh atom, which is consistent with a linear $\text{Rh-Rh}=\text{C}$ structure in which one carbene ligand binds to the axial site of the $\text{Rh}_2(\text{tpa})_4$ molecule. Models with four, five, or six C or O scatterers bound per Rh led to poorer fits to the data (30).

We used density functional theory (DFT) to investigate the electronic structure of **3**. We examined both **3** and **3a**, a model in which unsubstituted acetate replaced the bulky triphenylacetate ligands (Fig. 1B). Geometry optimization of either **3** or **3a** yielded a structure in good agreement with the experimental data available for **3** (Table 1). Greater molecular detail is available from these optimized geometries. For example, the EXAFS data can only be refined with an average Rh-C or Rh-O distance of 2.037(2) Å, whereas the optimized structure of **3** indicates individual Rh-O and $\text{Rh}=\text{C}$ distances of 2.04 Å and 1.97 Å, respectively. The weighted average of these distances, 2.03 Å, is in excellent agreement with the EXAFS result.

Other experimental observables that have been calculated for **3a** include the vibrations observed for **3** by rR spectroscopy and the ^{13}C NMR chemical shift and splitting for the carbene C atom. As seen in Table 1, these calculated data agree well with the experimentally observed values. The coupling constant $J_{\text{Rh-C}}$ is predicted accurately by the calculations because the Rh center is fully coordinated. The two-bond Rh-C coupling is likely overestimated by the gas-phase calculations. Mechanisms have been identified previously that reduce metal-ligand J coupling constants in the case of axial coordination by solvent (31, 32). The calculated vibrational frequencies reflect the typical overestimation by DFT calculations (33), but the magnitudes of the isotopic shifts are reproduced to within 1 cm^{-1} . The UV-Vis feature shown experimentally in Fig. 2C is also reproduced reasonably well by time-dependent DFT (fig. S10).

Bonding within the $\text{Rh-Rh}=\text{C}$ framework has been discussed in previous reports (23, 34–37), although never before could the electronic structure be substantiated by direct comparison to experimental data. Bonding along the Rh-Rh-C chain follows the three-center orbital paradigm described by Nakamura *et al.* (23) and Berry (37); this analysis allows for a useful explanation

Table 1. Comparison of experimental and calculated properties of 3/3a.

Method	Property	Experiment	DFT
		3	3/3a
EXAFS (Å)	Rh–Rh	2.434(2)	2.412/2.459
	Rh–O _{carboxylate}	—*	2.041/2.062
	Rh–C _{carbene}	—*	1.972/2.018
	Average Rh–C/O	2.037(2)*	2.027/2.040
		3	3a
		$^{12}\text{C}/^{13}\text{C}/\Delta^\dagger$	$^{12}\text{C}/^{13}\text{C}/\Delta^\dagger$
Resonance Raman vibrations (cm^{-1})	Mode 1‡	643.8/628.3/15.5	656.4/641.7/14.7
	Mode 2‡	889.9/876.5/13.4	913.0/898.8/14.2
	Mode 3‡	909.2/900.1/9.1	924.5/916.4/8.1
		3	3a
^{13}C NMR (CDCl ₃)	δ	240 ppm	218–270 ppm§
	$J_{\text{Rh-C}}$	27.3 Hz; not observed§	26.3–29.3 Hz§ 10.0–13.3 Hz§

*Only an average Rh-O or Rh-C distance could be refined from EXAFS data. $^\dagger\Delta = \nu(^{12}\text{C}) - \nu(^{13}\text{C})$; ν , energy of vibrational mode. ‡ Depictions of these vibrational modes are given in fig. S3. § The calculated NMR parameters are somewhat sensitive to the choice of functional, as further discussed in the supplementary materials. The second predicted coupling constant is to the distal Rh atom. This coupling is not resolved under our experimental conditions. ||In CD_2Cl_2 , $\delta = 242$ ppm and $J_{\text{Rh-C}} = 26.5$ Hz.

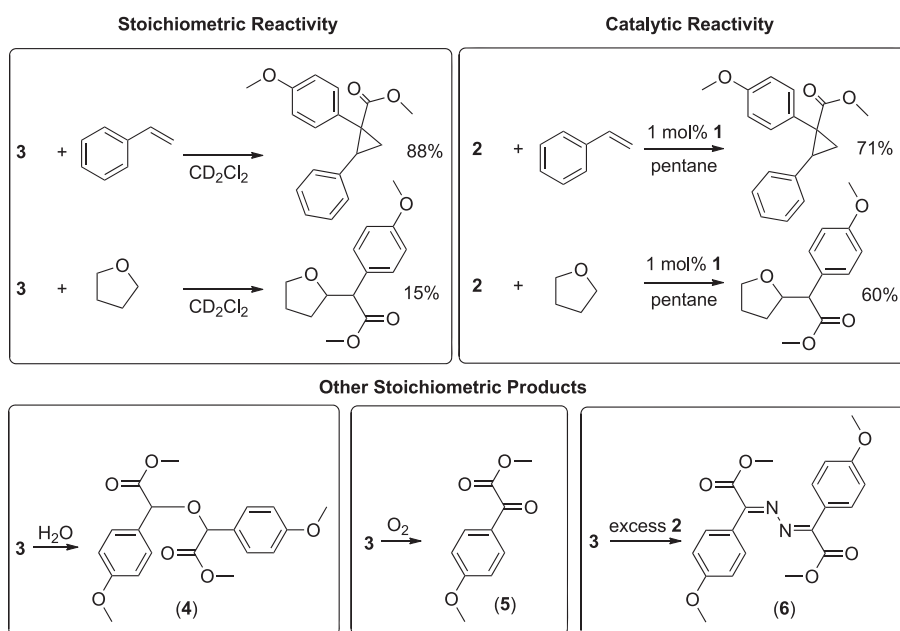


Fig. 3. Comparison of stoichiometric and catalytic reactivity for donor-acceptor carbenoid 3.

for the electrophilic reactivity of Rh₂ carbene species (see below), as well as an assignment of the low-energy visible absorption band observed for **3**. Weak Rh₂=C π bonding is accomplished by the formation of in-phase (π_1 , filled) and out-of-phase (π_2 , empty) combinations of the Rh₂ π^* orbital with the empty p orbital of the carbene unit (figs. S11 and S12). The π_2 orbital is the lowest unoccupied molecular orbital, and its polarization toward the carbene carbon gives this carbon atom empty p-orbital character that is the basis of the electrophilic reactivity of **3**. The Rh₂-carbene complex is electronically distinct from other more stable carbene complexes in that both the σ and π bonding components are substantially weakened by the electronic effects of the Rh–Rh bond (calculated Rh=C bond orders are ~ 0.7 to 0.8); this effect is probably responsible for kinetic lability of the carbene ligand in **3** that allows it to be so easily transferred to substrates. Based on this electronic structure analysis, the electronic absorption observed for **3** at ~ 720 nm may be assigned to a transition promoting an electron from π_1 to π_2 (fig. S11); the energy of this transition is indicative of the Rh–C π bond strength.

The catalytic reactivity of **1** with diazo compound **2** is parallel to other highly competent Rh₂ catalyst systems, as exhibited by high yields in both cyclopropanation and C–H functionalization reactions with various substrates, including styrene, tetrahydrofuran (THF), and even cyclohexane (**38**). For more challenging reactions such as insertion into nonactivated aliphatic C–H bonds, elevated temperatures are necessary for turnover. These conditions cannot be applied to stoichiometric solutions of **3**, as **3** can be generated only in chlorinated solvents, and heating induces rapid decomposition. Nonetheless, the stoichiometric reactivity of intermediate **3** with more active substrates such as styrene and THF is comparable to the respective catalytic reactions of **1**, supporting the proposed intermediacy of **3** in catalysis.

Nuclear magnetic resonance and mass spectroscopy have been used to identify products of stoichiometric reactions of **3** with other reagents: water, oxygen, and additional equivalents of diazoester **2** (Fig. 3). Both O–H bonds of water are susceptible to reaction with **3** to form **4**. Molecular oxygen is cleaved in the reaction with **3** to form the ketone product **5**. When **3** is introduced to additional equivalents of the diazo compound **2**, the carbene group is transferred to excess **2** to yield the dimeric azine compound **6**. Compounds **5** and **6** are known compounds that were identified by comparison of NMR data to authentic samples; **4** was unknown but has been identified by a combination of NMR and mass spectrometry. Compounds **4**, **5**, and **6** are all by-products identified in Rh₂-mediated catalysis. When no substrate is added, solutions of **3** in halocarbon solvents degrade slowly to form a multitude of organic species, likely products of radical reactions (*1*). However, the only Rh-

containing product after decomposition of **3** is **1**, as verified by NMR and EXAFS (the EXAFS data for **3-dec** are not significantly different from those of **1**).

The isolation of **3** has important implications to the field of catalysis: Direct evidence for this type of carbenoid intermediate has remained elusive since Paulisse *et al.* first reported the reaction between Rh₂(OAc)₄ (Ac, acetyl) and ethyl diazoacetate in 1973 (**39**). The characterization of this intermediate provides a firm foundation for future exploration of the reactivity of these highly selective carbenoid species. The key intermediate proposed in Rh₂-catalyzed carbenoid transformations is confirmed to be a genuine Rh₂ carbene complex. Perhaps more importantly, a broader understanding of bonding and reactivity of transition metal carbene complexes in the stability continuum from isolable Fischer- and Schrock-type carbenes to this less stable donor-acceptor carbenoid can be developed.

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Supplementary Materials

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Supplementary Text
Figs. S1 to S12
Tables S1 and S2
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Low Upper Limit to Methane Abundance on Mars

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By analogy with Earth, methane in the Martian atmosphere is a potential signature of ongoing or past biological activity. During the past decade, Earth-based telescopic observations reported “plumes” of methane of tens of parts per billion by volume (ppbv), and those from Mars orbit showed localized patches, prompting speculation of sources from subsurface bacteria or nonbiological sources. From in situ measurements made with the Tunable Laser Spectrometer (TLS) on Curiosity using a distinctive spectral pattern specific to methane, we report no detection of atmospheric methane with a measured value of 0.18 ± 0.67 ppbv corresponding to an upper limit of only 1.3 ppbv (95% confidence level), which reduces the probability of current methanogenic microbial activity on Mars and limits the recent contribution from extraplanetary and geologic sources.

Methane is the most abundant hydrocarbon in our solar system and is found in the atmospheres of several planets and satellites (1). On Earth, 90 to 95% of atmospheric methane is biologically produced, either from extant or fossil sources, and it is easy to identify and quantify with confidence by using spectroscopic methods (2). For Mars, three possible origins have been proposed: geologic, biotic, and exogenous (3–5). Over the past decade, there have been several reports of methane detection from Earth and from Mars orbit. Observations with the Canada-France-Hawaii Telescope (CFHT) found a global average value of 10 ± 3 parts per billion by volume (ppbv) (5). The Planetary Fourier Spectrometer (PFS) on the Mars Express (MEX) spacecraft found a global average abundance of 10 ± 5 ppbv (4), later updated (6) to 15 ppbv, with indications of discrete localized sources (4) and a summer time maximum of 45 ppbv in the north polar region. A search for methane from the Infrared Telescope Facility (IRTF) and the Keck-2 telescope reported methane release in plumes (7) from discrete sources in Terra Sabae, Nili Fossae, and Syrtis Major, with the largest plume containing 19,000 tons of CH₄ in March 2003; seasonal changes with a summer time maximum of ~45 ppbv near the equator were seen. Methane abundances later retrieved (8) from a second instrument in Mars orbit, the Thermal Emission Spectrometer (TES) of the Mars Global Surveyor (MGS), reported methane abundances as intermittently present (1999 to 2003), ranging from 5 to 60 ppbv in locations where favorable geological conditions such as residual geothermal activity (Tharsis and Elysium) and strong hydration (Arabia Terra) are expected. More recent observations report methane mixing ratios that

have diminished considerably since 2004 to 2006 to upper limits of 7 to 8 ppbv (9–11), suggesting a very short lifetime for atmospheric CH₄ and contradicting the MEX claim that methane persisted from 2004 to 2010. Ground-based observations favor episodic injection of methane in 1999 and 2003, 10 ppbv at Valles Marineris in February 2006 (9, 11), and <8 ppbv in January 2006 (10), 2009, and 2010; whereas orbital data from PFS and TES suggest a more regular behavior with latitudinal, seasonal, and interannual variabilities. At Curiosity’s Gale Crater landing site (4.5°S, 137°E), published maps of PFS data (6) show an increase from ~15 ppbv in fall to ~30 ppbv in winter, whereas the TES trend (8) is opposite: ~30 ppbv in fall and ~5 ppbv in winter.

The Tunable Laser Spectrometer (TLS) of the Sample Analysis at Mars (SAM) (12, 13) instrument suite on the Curiosity rover has a spectral resolution— 0.0002 cm^{-1} , which is far superior to those of the ground-based telescopic and orbiting spectrometers—that offers unambiguous identification of methane in a distinct fingerprint spectral pattern of three well-resolved adjacent ¹²CH₄ lines in the 3.3 μm band (Fig. 1). The in situ technique of tunable laser absorption in a closed sample cell is simple, noninvasive, and sensitive. TLS is a two-channel tunable laser spectrometer that uses both direct and second harmonic detection of infrared (IR) laser light. One laser source is a near-IR tunable diode laser at 2.78 μm that can scan two spectral regions containing CO₂ and H₂O isotopic lines that have been used to report ¹³C/¹²C, ¹⁸O/¹⁷O/¹⁶O, and D/H ratios in the Martian atmosphere (13). The second laser source is an interband cascade (IC) laser at 3.27 μm used for methane detection alone, scanning across seven rotational lines that includes the R(3) triplet used in this study (Fig. 1 and table S1). The IC laser beam makes 81 passes of a 20-cm-long sample cell of the Herriott design fitted with high-vacuum microvalves that allow evacuation with a turbomolecular pump for “empty cell” scans or filled to Mars ambient pressure (~8 mbar) for “full cell” runs. During data collection, the cell and other optics are kept at $47 \pm 3^\circ\text{C}$ by using a heater that thermally stabilizes the cell but is ramped up and

down within these temperature limits in order to increase gas sensitivity by spoiling the accumulation of optical interference fringes during the 2-min period of spectrum collection. Our methane determination is made by comparing the measured methane abundances in our sample cell when filled with Mars atmosphere with those of the same cell evacuated, as detailed in (14). The laser scans every second through the methane spectral region, and each spectrum is co-added on board to downlink sequential 2-min-averaged spectra during a given run of ~1 to 2 hours in duration. Typically, we recorded 26 2-min “empty cell” spectra followed by 26 2-min “full cell” spectra, then finally five additional 2-min empty cell spectra. For each 2-min spectrum, we retrieved methane abundances from three spectral lines (14) individually and combined the results so as to produce a weighted average value. By subtracting all retrieved abundances (full and empty cell) from the empty cell mean value for that sol (one Martian solar day) run, we were left with 31 differences for the empty cell and 26 for the full cell. For our statistical analysis, we analyzed the empty cell and full cell differences for all the sols taken as one data set (14). For sols 79, 81, 106, and 292, the foreoptics chamber contained residual terrestrial air (Table 1, pressures), including CH₄, that produced absorption line signals in the sample cell detector channel, as described in (14). For sols 306 and 313, the foreoptics was evacuated. Both the sample cell and foreoptics chamber have pressure and temperature sensors. This experiment has been repeated on six separate Martian sols to date (Martian sols 79, 81, 106, 292, 306, and 313 after landing in August 2012). The inlet to the TLS is a stainless steel tube (14) heated to 50°C and located on the rover side ~1 m above the Martian surface and was pointed at a variety of directions relative to the nominal wind direction. Mars atmospheric gas was ingested during the night for sols 79, 81, 106, 292, and 313 and during the day for sol 306 (Table 1). Our measurements correspond to southern spring (sols 79, 81, and 106) and mid-late summer (sols 292, 306, and 313) on Mars.

To date, we have no detection of methane. Individually (Table 1), each of our six data sets produces a mean methane value ranging from –2.2 to 1.7 ppbv. Combining the individual sol results with equal weighting yields a mean and SE of 0.11 ± 0.67 ppbv. Alternatively, combining all of the individual measurements from all sols yields a grand mean and SE of 0.18 ± 0.67 ppbv. At the 95% confidence level, either approach (14) yields an upper limit on Mars atmospheric methane of 1.3 ppbv. Curiosity’s low upper limit is not expected given observations only a few years ago of large methane plumes and calculations (7) that the plume dispersion should produce global values of ~6 ppbv after the 6-month period (3, 15) needed to mix uniformly across the planet, which would persist with a photochemical lifetime of several hundred years (3, 5, 16).

Before Curiosity’s landing on Mars in August 2012, observational evidence for methane on

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Mars was questioned in the published literature (15, 17, 18). Contradictions were noted between the locations of maxima reported from ground-based observations and maps inferred by PFS and TES from Mars orbit. The plume results (7) were questioned (17) on the basis of a possible misinterpretation from methane lines whose positions coincided with those of terrestrial isotopic ¹³CH₄ lines. Krasnopolsky (19) argued that cometary and volcanic contributions were not sufficient to explain high methane abundances, calculating a cometary contribution of only ~0.1 ppbv and noting the lack of current volcanism, lack of hot spots in thermal imaging (20), and the extremely

low upper limit for Mars SO₂ (9, 21) that in Earth's volcanic emissions is orders of magnitude more abundant than CH₄ (5). The very short methane lifetime of 0.4 to 4 years derived from the 2003–2006 observations (7) requires powerful destruction mechanisms that have not been identified to date. Although models have been proposed for rapid removal of methane by oxidants—such as hydrogen peroxide and perchlorates, or by superoxides derived from their mineral reactions (22–24) and directly by electric fields generated in dust devils (25)—there remains no evidence for their existence at Mars. Moreover, it has not been demonstrated that any of these

processes can reduce the lifetime of methane by the required factor of 100 or more compared with its photochemical lifetime. Our reported upper limit of 1.3 ppbv is substantially lower than the methane abundances reported from Mars remote sensing spacecraft observations and those from Earth telescopic observations, including both the earlier high values of typically tens of ppbv and the more recently reported upper limits of 7 to 8 ppbv (9, 10). Although TLS samples only the very lowest part (~1 m) of the Mars atmosphere as compared with those of the other observations that are vertical column-integrated results, the atmospheric scale height (~10 km) and mixing

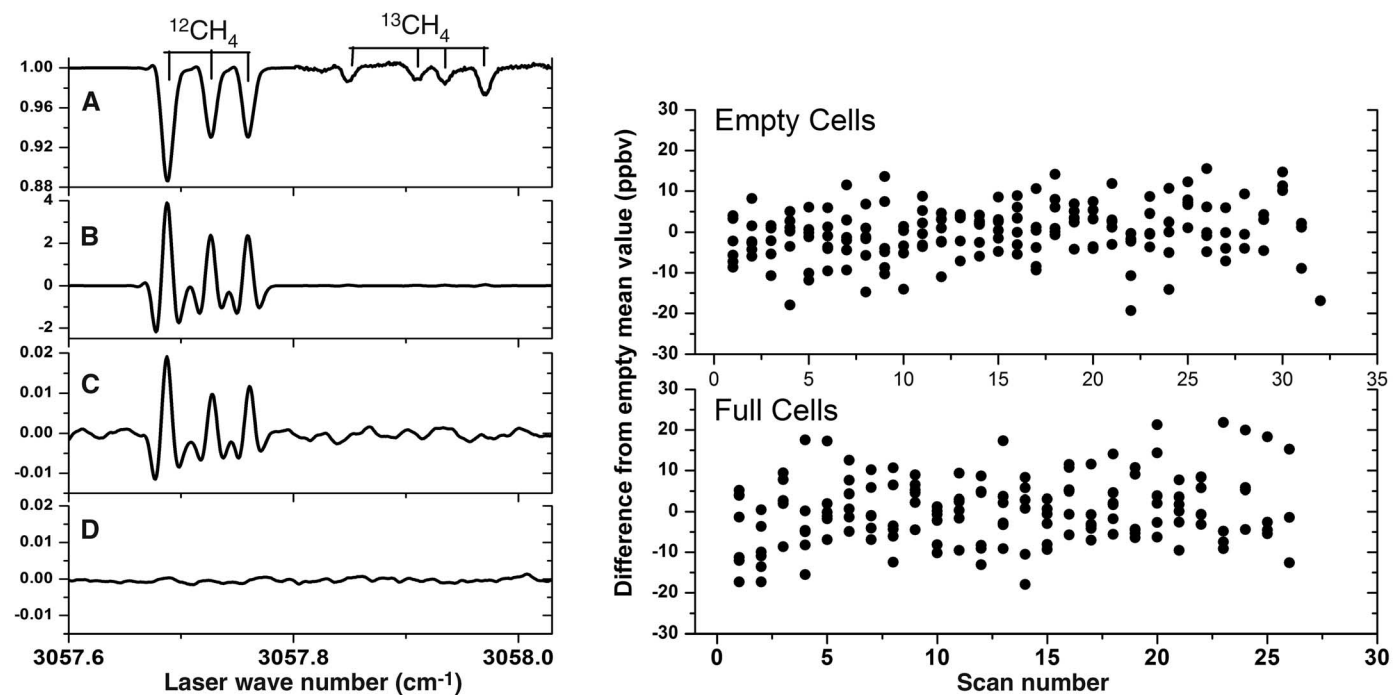


Fig. 1. The TLS-SAM methane measurements. (Left) Examples of flight spectra downloaded from Curiosity. (A) Spectrum recorded during an unrelated Evolved Gas Analysis (EGA) run (14) showing location of ¹²CH₄ and ¹³CH₄ lines, in which the second half has been vertically expanded by ×20 to show the weaker ¹³CH₄ lines. (B) Same as (A) but second harmonic (2f) spectrum (14), without vertical expansion. (C) Averaged full cell 2f spectrum for sol 106 (nighttime ingest), with foreoptics contribution (14). (D) Averaged

full cell 2f spectrum for sol 306 (daytime ingest), with foreoptics evacuated. [Spectra (A) and (B) are shown here in part because they were taken after the atmospheric runs and show that our CH₄ lines have not moved and that the instrument continued to work well with consistent capability to detect methane.] (Right) Individual 2-min data points from 6 sols. (Top) Empty cell data with mean value of 0.0 ppbv. (Bottom) Full cell data with mean value of 0.18 ppbv.

Table 1. Curiosity SAM-TLS methane measurements at Gale Crater (4.5°S, 137.4°E) over an 8-month period. SEM, standard error from the mean; Ls, solar longitude.

Martian sol after landing on 6 August 2012	Earth date	Ls (degrees)	Gas ingest time/cell pressure (mbar)/foreoptics pressure (mbar)	Mean value ± 1 SEM (ppbv)
79	25 October 2012	195.0	Night/8.0/11.5	1.62 ± 2.03
81	27 October 2012	196.2	Night/8.0/11.5	1.71 ± 2.06
106	27 November 2012	214.9	Night/8.5/10.9	−0.55 ± 1.45
292	1 June 2013	328.6	Night/8.7/9.2	0.60 ± 1.74
306	16 June 2013	336.5	Day/8.1/0.0	−2.21 ± 0.94
313	23 June 2013	340.5	Night/8.7/0.0	−0.50 ± 0.94
Mean of individual sol results	0.11 ± 0.56			
Mean for entire aggregated data set	0.18 ± 0.67			

time (approximately a few months) suggests that our measured upper limit is representative of the global mean background level. With an expected photochemical lifetime of methane in the Martian atmosphere of hundreds of years (3, 5, 16), there currently remains no accepted explanation (15, 17) for the existence and distribution of the reported plumes nor of the apparent disappearance of methane over the past few years. Our result sets an upper limit that is ~6 times lower than other recent measurements and greatly reduces the probability of substantial methanogenic microbial activity on Mars and recent methane production through serpentinization or from exogenous sources, including meteoritic, interplanetary dust and cometary infall.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S5
Tables S1 to S3
MSL Science Team Author List
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Genomically Recoded Organisms Expand Biological Functions

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We describe the construction and characterization of a genomically recoded organism (GRO). We replaced all known UAG stop codons in *Escherichia coli* MG1655 with synonymous UAA codons, which permitted the deletion of release factor 1 and reassignment of UAG translation function. This GRO exhibited improved properties for incorporation of nonstandard amino acids that expand the chemical diversity of proteins in vivo. The GRO also exhibited increased resistance to T7 bacteriophage, demonstrating that new genetic codes could enable increased viral resistance.

The conservation of the genetic code permits organisms to share beneficial traits through horizontal gene transfer (1) and enables the accurate expression of heterologous genes in nonnative organisms (2). However, the

common genetic code also allows viruses to hijack host translation machinery (3) and compromise cell viability. Additionally, genetically modified organisms (GMOs) can release functional DNA into the environment (4). Virus resistance (5) and biosafety (6) are among today's major unsolved problems in biotechnology, and no general strategy exists to create genetically isolated or virus-resistant organisms. Furthermore, biotechnology has been limited by the 20 amino acids of the canonical genetic code, which use all 64 possible triplet codons, limiting efforts to expand the chemical properties of proteins by means of nonstandard amino acids (NSAAs) (7, 8).

Changing the genetic code could solve these challenges and reveal new principles that explain how genetic information is conserved, encoded, and exchanged (fig. S1). We propose that genomically recoded organisms (GROs, whose codons have been reassigned to create an alternate genetic code) would be genetically isolated from natural organisms and viruses, as horizontally transferred genes would be mistranslated, pro-

ducing nonfunctional proteins. Furthermore, GROs could provide dedicated codons to improve the purity and yield of NSAA-containing proteins, enabling robust and sustained incorporation of more than 20 amino acids as part of the genetic code.

We constructed a GRO in which all instances of the UAG codon have been removed, permitting the deletion of release factor 1 (RF1; terminates translation at UAG and UAA) and, hence, eliminating translational termination at UAG codons. This GRO allows us to reintroduce UAG codons, along with orthogonal translation machinery [i.e., aminoacyl-tRNA synthetases (aaRSs) and tRNAs] (7, 9), to permit efficient and site-specific incorporation of NSAAs into proteins (Fig. 1). That is, UAG has been transformed from a nonsense codon (terminates translation) to a sense codon (incorporates amino acid of choice), provided the appropriate translation machinery is present. We selected UAG as our first target for genome-wide codon reassignment because UAG is the rarest codon in *Escherichia coli* MG1655 (321 known instances), prior studies (7, 10) demonstrated the feasibility of amino acid incorporation at UAG, and a rich collection of translation machinery capable of incorporating NSAAs has been developed for UAG (7).

We used an in vivo genome-editing approach (11), which is more efficient than de novo genome synthesis at exploring new genotypic landscapes and overcoming genome design flaws. Although a single lethal mutation can prevent transplantation of a synthetic genome (12), our approach allowed us to harness genetic diversity and evolution to overcome any potential deleterious mutations at a cost considerably less than de novo genome synthesis (supplementary text section B, "Time and cost"). In prior work, we used multiplex automated genome engineering [MAGE (13)] to remove all known UAG codons in groups of 10

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across 32 *E. coli* strains (11), and conjugative assembly genome engineering [CAGE (11)] to consolidate these codon changes in groups of ~80 across four strains. In this work, we overcome technical hurdles (supplementary text) to complete the assembly of the GRO and describe the biological properties derived from its altered genetic code.

The GRO [C321.ΔA, named for 321 UAG→UAA conversions and deletion of *prfA* (encodes RF1, Table 1)] and its RF1⁺ precursor (C321) exhibit normal prototrophy and morphology (fig. S2), with 60% increased doubling time compared with *E. coli* MG1655 (table S1). Genome sequencing [GenBank accession CP006698] confirmed that all 321 known UAGs were removed from its genome and that 355 additional mutations were acquired during construction (10⁻⁸ mutations per base pair per doubling over ~7340 doublings; fig. S3 and tables S2 to S4). Although maintaining the *E. coli* MG1655 genotype was not a primary goal of this work, future applications requiring increased genome stability could exploit reversible switching of *mutS* function (14) to reduce off-target mutagenesis. CAGE improved the fitness of several strains in the C321 lineage (fig. S3), implicating off-target mutations in the reduced fitness.

C321.ΔA exhibited improved performance compared with previous strategies for UAG codon reassignment (15, 16), permitting the complete reassignment of UAG from a stop codon to a sense codon capable of incorporating NSAAs into proteins. One previous strategy used a variant of release factor 2 (RF2) that exhibits enhanced UAA termination (16) and weak UAG termination (17). The second strategy substituted a UAA stop codon in each of the seven essential genes naturally terminating with UAG (table S5) and reduced ribosome toxicity by efficiently incorporating amino acids at the remaining 314 UAGs (15). For comparative purposes, we used MAGE to create strains C0.B*.ΔA::S [expresses enhanced RF2 variant (16)], C7.ΔA::S (UAG changed to UAA in seven essential genes), and C13.ΔA::S [UAG changed to UAA in seven essential genes plus six nonessential genes (table S5)] (Table 1). C refers to the number of codon changes, while A and B refer to *prfA* (RF1) and *prfB* (RF2) manipulations, respectively. In contrast to previous work (15), we deleted RF1 in these strains without introducing a UAG suppressor, perhaps because near-cognate suppression is increased in *E. coli* MG1655 (18). Nevertheless, these strains exhibited a strong selective pressure to acquire UAG suppressor mutations (see below).

To assess the fitness effects of RF1 removal and UAG reassignment, we measured the doubling time and maximum cell density of each strain (table S1 and fig. S4). We found that C321 was the only strain for which RF1 removal and UAG reassignment was not deleterious (Fig. 2). Because we did not modify RF2 to enhance UAA termination (16), this confirms that RF1 is essential only for UAG translational termination and not for UAA termination or other essential cellular

functions. By contrast, RF1 removal significantly impaired fitness for C0.B*.ΔA::S, and codon reassignment exacerbated this effect (Fig. 2 and fig. S5), probably because NSAA incorporation outcompeted the weak UAG termination activity (17) exerted by the RF2 variant (16). C7.ΔA::S and C13.ΔA::S also exhibited strongly impaired fitness, likely due to more than 300 nonessential UAG codons stalling translation in the absence of RF1-mediated translation at UAG codons (15); accordingly, *p*-acetylphenylalanine (pAcF) incorporation partially alleviated this effect (Fig. 2). However, not all NSAAs improved fitness in partially recoded strains; phosphoserine (Sep) impairs fitness in similar strains (19), perhaps by causing proteome-scale misfolding. Together, these results indicate that only the complete removal of all instances of the UAG codon overcomes these deleterious effects; therefore, it may be the only scalable strategy for sustained NSAA translation and for complete reassignment of additional codons.

We tested the capacity of our recoded strains to efficiently incorporate NSAAs [pAcF, *p*-azidophenylalanine (pAzF), or 2-naphthalalanine (NapA)] into green fluorescent protein (GFP) variants containing zero, one, or three UAG codons (Fig. 3 and fig. S6). In the presence of NSAAs, the RF1⁺ strains efficiently read through variants containing three UAGs, demonstrating that the episomal pEVOL translation system, which expresses an aaRS and tRNA that incorporate a NSAA at UAG codons (9), is extremely active and strongly outcompetes RF1. In the absence of NSAAs, the RF1⁺ strains exhibited detectable amounts of near-cognate suppression (18) of a single UAG. C321.ΔA::S exhibited strong expression of UAG-containing GFP variants only in the presence of the correct NSAA, whereas C7.ΔA::S and C13.ΔA::S displayed read-through of all three UAG codons even in the absence of NSAAs, suggesting efficient incorporation of natural amino acids at native UAGs (17). Mass spectrometry indicated that C13.ΔA::S incorporated Gln, Lys, and Tyr at UAG codons. DNA sequencing in C7.ΔA::S and C13.ΔA::S revealed UAG suppressor mutations in *glnV*, providing direct genetic evidence of Gln suppression observed by Western blot (Fig. 3A) and mass spectrometry (table S13). C0.B*.ΔA::S displayed truncated GFP variants corresponding with UAG termination in the absence of RF1 (17) (Fig. 3A).

We directly investigated the impact of pAcF and Sep incorporation on the proteomes (Fig. 3B) (20) of our panel of strains (Table 1) using mass spectrometry (tables S6 to S12). No Sep-containing peptides were observed for EcNR2, illustrating that RF1 removal is necessary for NSAA incorporation by the episomal phosphoserine system (21), which is an inefficient orthogonal translation machinery (19) (Fig. 3C and table S10). By contrast, we observed NSAA-containing peptides in unrecoded (C0.B*.ΔA::S) and partially recoded (C13.ΔA::S) strains, and not the GRO (C321.ΔA::S), which lacks UAGs in its genome

Wild type UAG denotes translation stop

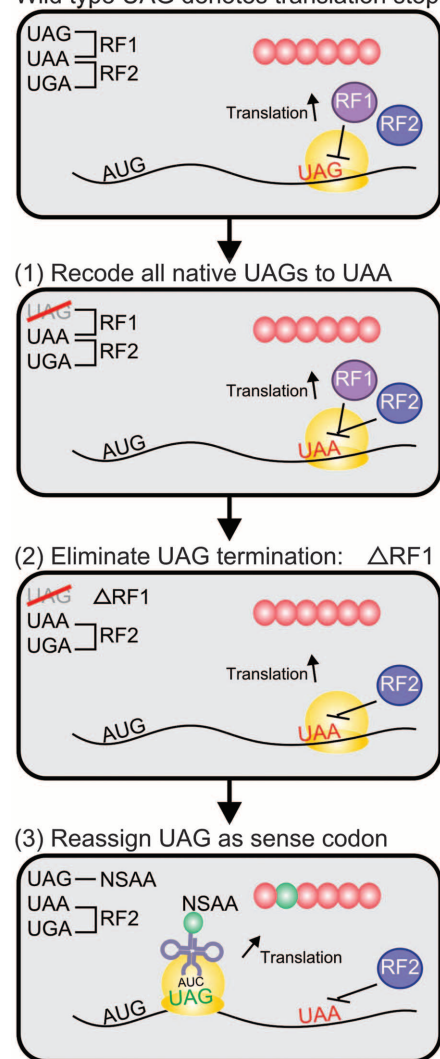


Fig. 1. Engineering a GRO with a reassigned UAG codon. Wild-type *E. coli* MG1655 has 321 known UAG codons that are decoded as translation stops by RF1 (for UAG and UAA). (1) Remove codons: converted all known UAG codons to UAA, relieving dependence on RF1 for termination. (2) Eliminate natural codon function: abolished UAG translational termination by deleting RF1, creating a blank codon. (3) Expand the genetic code: introduced an orthogonal aminoacyl-tRNA synthetase (aaRS) and tRNA to reassign UAG as a dedicated sense codon capable of incorporating nonstandard amino acids (NSAAs) with new chemical properties.

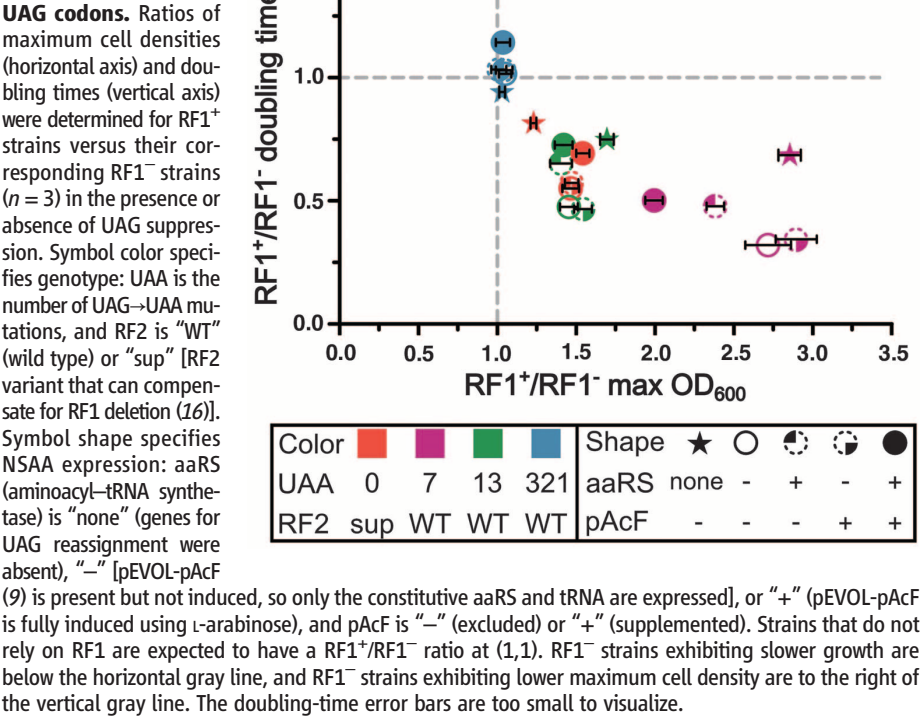
(Fig. 3, B and C, fig. S7, and tables S6 to S12). Such undesired incorporation of NSAAs (or natural amino acids) likely underlies the fitness impairments observed for C0.B*.ΔA::S, C7.ΔA::S, and C13.ΔA::S. In contrast to the other RF1⁺ strains, C321.ΔA::S demonstrated equivalent fitness to its RF1⁺ precursor (Fig. 2) and efficiently expressed all GFP variants without incorporating NSAAs at unintended sites (Figs. 2 and 3 and fig. S6). Therefore, complete UAG removal is the only strategy that provides a devoted codon for

Table 1. Recoded strains and their genotypes.

Strain*	Essential codons changed†	Total codons changed‡	Previously essential codon functions manipulated§	Expected (obs.) UAG translation function
EcNR2	0	0	None	Stop
C0.B [¶]	0	0	<i>prfB</i> [#]	Stop
C0.B [¶] .ΔA::S	0	0	<i>prfB</i> [#] Δ <i>prfA</i> :: <i>spec</i> ^R	None (stop [¶])
C7	7	7	None	Stop
C7.ΔA::S	7	7	Δ <i>prfA</i> :: <i>spec</i> ^R	None (sup)
C13	7	13	None	Stop
C13.ΔA::S	7	13	Δ <i>prfA</i> :: <i>spec</i> ^R	None (sup)
C321	7	321	None	Stop
C321.ΔA::S	7	321	Δ <i>prfA</i> :: <i>spec</i> ^R	None (nc)
C321.ΔA::T	7	321	Δ <i>prfA</i> :: <i>tolC</i>	None (nc)
C321.ΔA	7	321	Δ <i>prfA</i>	None (nc)

*All strains are based on EcNR2 [*E. coli* MG1655 Δ(*ybhB-bioAB*)::[*λ*cl857 N(*cro-ea59*)::*tetR-bla*] Δ*mutS*::*cat*], which is mismatch repair deficient (Δ*mutS*) to achieve high-frequency allelic replacement; C0 and C321 strains are Δ*mutS*::*zeo*^R; C7 and C13 strains are Δ*mutS*::*tolC*; C7, C13, and C321 strains have the endogenous *tolC* deleted, making it available for use as a selectable marker. Spectinomycin resistance (S) or *tolC* (T) were used to delete *prfA* (A). Bacterial genetic nomenclature describing these strains includes :: (insertion) and Δ (deletion). †Out of a total of 7. ‡Out of a total of 321. §*prfA* encodes RF1, terminating UAG and UAA; *prfB* encodes RF2, terminating UGA and UAA; *prfB*[#] is an RF2 variant (T246A, A293E, and removed frameshift) exhibiting enhanced UAA termination (16) and weak UAG termination (17). ||Observed translation function: Stop, expected UAG termination; stop[¶], weak UAG termination from RF2 variant; sup, strong selection for UAG suppressor mutations; nc, weak near-cognate suppression (i.e., reduced expression compared to C7.ΔA::S and C13.ΔA::S) in the absence of all other UAG translation function.

Fig. 2. Effects of UAG reassignment at natural UAG codons.



plug-and-play NSAA incorporation without impairing fitness (Figs. 2 and 3). To determine whether this GRO can obstruct viral infection, we challenged RF1⁻ strains with bacteriophages T4 and T7. Viruses rely on their host to express proteins necessary for propagation. Because hosts with altered genetic codes would mistranslate viral proteins (3), recoding may provide a general mechanism for resistance to all natural viruses. Given that UAG codons occur rarely and only at the end of genes, we did not expect UAG reassignment to result in broad phage resistance. Although the absence of RF1

did not appear to affect T4 (19 of 277 stop codons are UAG), it significantly enhanced resistance to T7 (6 of 60 stop codons are UAG) (Fig. 4). RF1⁻ hosts produced significantly smaller T7 plaques independent of host doubling time (Fig. 4A and fig. S8). The only exception was C0.B*ΔA::S, which produced statistically equivalent plaque sizes regardless of whether RF1 was present (Fig. 4A and table S14). Consistent with the observation that the modified RF2 variant could weakly terminate UAG [(17) and herein], our results suggest that C0.B*ΔA::S terminates UAG codons well enough to support normal T7 infection.

Given that plaque area and phage fitness (doublings per hour) do not always correlate, we confirmed that T7 infection is inhibited in RF1⁻ hosts by comparing T7 fitness and lysis time in C321 versus C321.ΔA (Fig. 4B). Phage fitness (doublings per hour) is perhaps the most relevant measure for assessing phage resistance because it indicates how quickly a log-phase phage infection expands (22). We found that T7 fitness was significantly impaired in strains lacking RF1 (*P* = 0.002), and kinetic lysis curves (fig. S9) confirmed that lysis was significantly delayed in the absence of RF1 (*P* < 0.0001, Fig. 4B). Meanwhile, one-step growth curves (fig. S10) indicated that burst size (average number of phages produced per lysed cell) in RF1⁻ hosts was also reduced by 59% (±9%), and phage packaging was delayed by 30% (±2%) (table S15). We hypothesize that ribosome stalling at the gene 6 (T7 exonuclease) UAG explains the T7 fitness defect in RF1⁻ hosts, whereas T4 may not possess a UAG-terminating essential gene with a similar sensitivity (supplementary text). Abolishing the function of additional codons could block the translation of viral proteins and prevent infections entirely. Using multiplex genome editing, we removed all instances of the UAG codon and reassigned its function in the genome of a living cell. The resulting GRO possesses a devoted UAG sense codon for robust NSAA incorporation that is suitable for industrial protein production. GROs also establish the basis for genetic isolation and virus resistance, and additional recoding will help fully realize these goals—additional triplets could be reassigned, unnatural nucleotides could be used to produce new codons (23), and individual triplet codons could be split into several unique quadruplets (8, 24) that each encode their own NSAA. In an accompanying study (25), we show that 12 additional triplet codons may be amenable to removal and eventual reassignment in *E. coli*. However, codon usage rules are not fully understood, and recoded genome

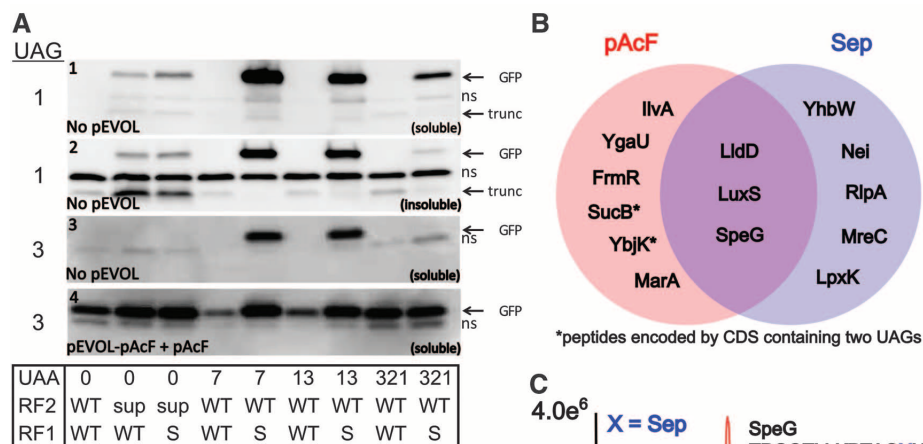


Fig. 3. NSAA incorporation in GROs. (A) Western blots demonstrate that C0.B*.ΔA::S terminates at UAG in the absence of RF1 and that C7.ΔA::S and C13.ΔA::S have acquired natural suppressors that allow strong NSAA-independent read-through of three UAG codons. When pAcF was omitted, one UAG reduced the production of full-length GFP, and three UAGs reduced production to undetectable levels for all strains except C7.ΔA::S and C13.ΔA::S, demonstrating that undesired near-cognate suppression (18) is weak for most strains even when RF1 is inactivated. However, all strains show efficient translation through three UAG codons when pAcF is incorporated. Western blots were probed with an antibody to GFP that recognizes an N-terminal epitope. UAA is the number of UAG→UAA mutations; RF2 is "WT" (wild type) or "sup" [RF2 variant that can compensate for RF1 deletion (16)]; RF1 is "WT" (wild type) or "S" (*ΔprfA::spec^R*). "GFP" is full-length GFP; "trunc" is truncated GFP from UAG termination and is enriched in the insoluble fraction; "ns" indicates a nonspecific band. (B) Venn diagram representing NSAA-containing peptides detected by mass spectrometry in C0.B*.ΔA::S when UAG was reassigned to incorporate *p*-acetylphenylalanine (pAcF, red) or phosphoserine (Sep, blue). No NSAA-containing peptides were identified in C321.ΔA::S. Asterisk (*) indicates coding DNA sequence possessing two tandem UAG codons. (C) Extracted ion chromatograms are shown for UAG suppression of the SpeG peptide to investigate Sep incorporation in natural proteins. Peptides containing Sep were only observed in C0.B*.ΔA::S, C7.ΔA::S, and C13.ΔA::S, as Sep incorporation was below the detection limit in EcNR2 (RF1⁺), and *speG* was recoded in C321.ΔA::S.

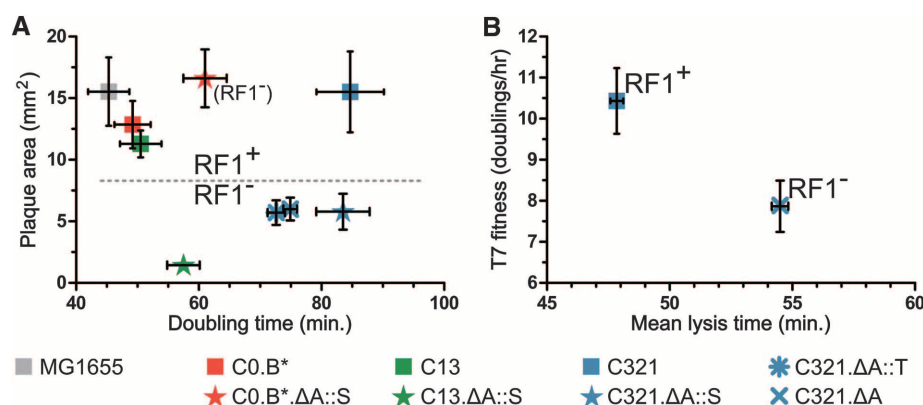


Fig. 4. Bacteriophage T7 infection is attenuated in GROs lacking RF1. RF1 (*prfA*) status is denoted by symbol shape: (■) wt *prfA* (WT); (★) *ΔprfA::spec^R* (ΔA::S); (✱) *ΔprfA::tolC* (ΔA::T); and (X) a clean deletion of *prfA* (ΔA). (A) RF1 status affects plaque area (Kruskal-Wallis one-way analysis of variance, $P < 0.001$), but strain doubling time does not (Pearson correlation, $P = 0.49$). Plaque areas (mm²) were calculated with ImageJ, and means $\pm$ 95% confidence intervals are reported ($n > 12$ for each strain). In the absence of RF1, all strains except C0.B*.ΔA::S yielded significantly smaller plaques, indicating that the RF2 variant (16) can terminate UAG adequately to maintain T7 fitness. A statistical summary can be found in table S14. (B) T7 fitness (doublings/hour) (22) is impaired ($P = 0.002$) and mean lysis time (min) is increased ($P < 0.0001$) in C321.ΔA compared to C321. Significance was assessed for each metric by using an unpaired *t* test with Welch's correction.

designs are likely to contain unknown lethal elements. Thus, it will be necessary to sample vast genetic landscapes, efficiently assess phenotypes arising from individual changes and their combinations, and rapidly iterate designs to change the genetic code at the genome level.

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Supplementary Materials

www.sciencemag.org/content/342/6156/357/suppl/DC1
Materials and Methods
Figs. S1 to S22
Tables S1 to S37
References (26–70)

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Probing the Limits of Genetic Recoding in Essential Genes

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Engineering radically altered genetic codes will allow for genomically recoded organisms that have expanded chemical capabilities and are isolated from nature. We have previously reassigned the translation function of the UAG stop codon; however, reassigning sense codons poses a greater challenge because such codons are more prevalent, and their usage regulates gene expression in ways that are difficult to predict. To assess the feasibility of radically altering the genetic code, we selected a panel of 42 highly expressed essential genes for modification. Across 80 *Escherichia coli* strains, we removed all instances of 13 rare codons from these genes and attempted to shuffle all remaining codons. Our results suggest that the genome-wide removal of 13 codons is feasible; however, several genome design constraints were apparent, underscoring the importance of a strategy that rapidly prototypes and tests many designs in small pieces.

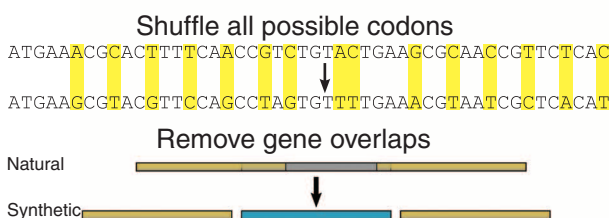
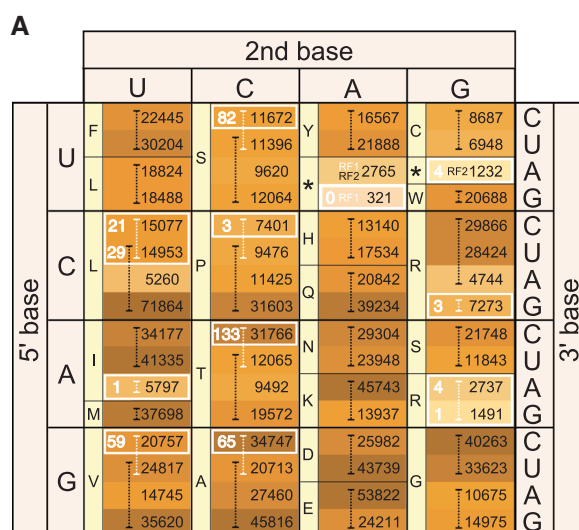
The canonical genetic code is nearly universal (1), allowing natural organisms to share beneficial traits via horizontal gene transfer. Genetically modified organisms also share this code, rendering them susceptible to viruses and capable of releasing recombinant genetic material [e.g., resistance genes (2)] into

the environment. By redefining the genetic code, we hope to produce genomically recoded organisms (GROs) that are safe and useful.

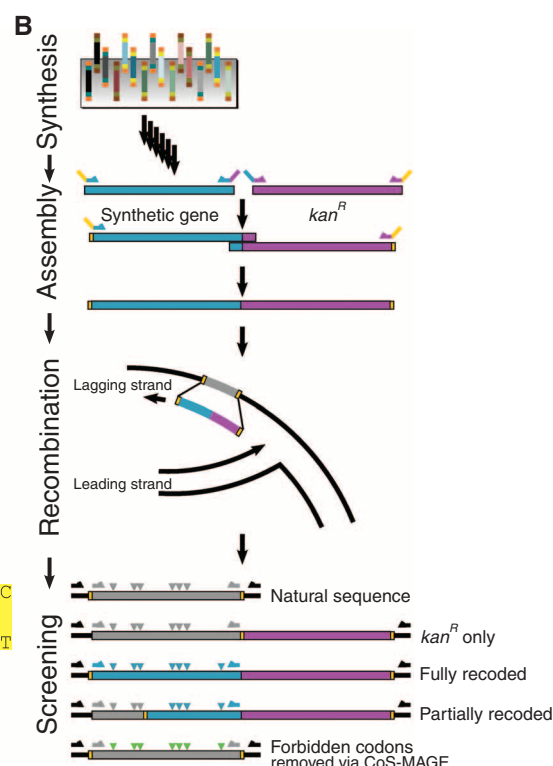
In separate work, we have completely reassigned the UAG codon in *Escherichia coli* MG1655 (3). UAG was chosen for its rarity and simplicity of function, but our results (3) reinforce

that sense codons must also be reassigned to achieve robust genetic isolation, broad virus resistance, and expanded chemical versatility (4). However, sense codon reassignment poses a considerable challenge given that codon usage can strongly affect gene regulation (5), ribosome spacing (6, 7), translation efficiency (7, 8), translation levels (9), translation accuracy (10), and protein folding (11, 12). Furthermore, DNA/RNA motifs can provide additional noncoding functions such as regulating translation initiation via 5' mRNA secondary structure (13), sharing sequence with overlapping small RNAs (14), pausing the ribosome at internal Shine-Dalgarno sequences (15), and regulating mRNA localization (16). Therefore, it is difficult to predict the effects of a given codon change, and these factors may substantially constrain the malleability of the genome.

Fig. 1. Codon reassignment across 42 essential genes. (A) *E. coli* MG1655 codon usage heat map; brightness increases as codon usage decreases. Black numbers are total codon usage based on NC_000913.2 (National Center for Biotechnology Information, 1 September 2011). The anticodon specificities (29, 30) are illustrated as dashed brackets; white indicates anticodons that were targeted for eventual removal. Amino acids are indicated in the yellow side bars. White boxes denote the 13 forbidden codons, and white numbers report how many instances of each codon were in the panel of 42 targeted essential genes. All 405 instances of these forbidden codons were successfully recoded across 80 *E. coli* strains. Additionally, all possible codons were swapped



to synonymous codons, and gene overlaps were removed by duplication (bottom). (B) Strategy for recoding essential genes. Recoded genes (blue rectangles) were synthesized from Agilent Oligonucleotide Library Synthesis arrays (24), then transcriptionally fused to *kan^R* (purple rectangles) by isothermal assembly (25). These cassettes were recombined into EcNR2 [*E. coli* MG1655 $\Delta(ybhB-bioAB)::\lambda cl857N(cro-ea59)::tetR-bla) \Delta mutS::cat$] using λ Red (26), and recombinants were selected on kanamycin. Putative recombinants were screened with three sets of primers: Wild-type primers (gray) hybridize specifically to the natural gene sequence, mutant primers (blue) hybridize specifically to the recoded gene sequence, and boundary primers (black) hybridize to the



surrounding genomic DNA. Desired recombinants were detected by polymerase chain reaction and then verified by Sanger sequencing. We found that *kan^R* ("*kan^R* only") could be inserted downstream of all genes except for *rplO* without causing major deleterious effects. We attempted to replace all 42 natural genes with radically recoded versions ("Fully recoded"; blue rectangles and triangles are recoded sequence). To coarsely map problematic design elements in the failed cassettes, we prepared cassettes that preserved natural sequence at the N terminus ("Partially recoded"; gray rectangles and triangles are natural sequence). Finally, all remaining forbidden codons were recoded with CoS-MAGE (green triangles) and confirmed with Sanger sequencing.

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However, despite the myriad mechanisms by which swapping synonymous codons could be deleterious, efforts to express a codon-randomized *Klebsiella* nitrogenase gene cluster in *E. coli* have been successful, albeit with reduced activity compared with wild type (17).

Although such information is critical for reassigning the genetic code, genome-wide codon essentiality has largely been unexplored, perhaps due to the substantial degree of genetic modification necessary for addressing such questions. For example, the complete removal of 13 codons corresponding to the least frequently used anticodons (Fig. 1A and supplementary text) will require 155,224 changes in *E. coli* MG1655, several of which may not be tolerated. Although it has never been attempted, de novo genome design, synthesis, and transplantation (17) seem unlikely to produce a viable genome bearing this unprecedented number of potentially deleterious changes. Indeed, lethal genetic elements have been difficult to identify and eliminate using de novo genome transplantation (16). Therefore, we have developed in vivo multiplex genome editing technologies (18, 19) to rapidly prototype and manufacture genomes. Our approach exploits diversity and natural selection and is highly amenable to our goal of testing the flexibility of synonymous codon choices as they pertain to reassigning the genetic code.

To address this question, we attempted to individually recode 42 essential genes, including all 41 essential ribosomal protein-coding genes (20) and *prfB*, which relies on a programmed frameshift for proper translation (21). Because expression level correlates strongly with codon usage bias (9), the highly expressed and tightly regulated (22, 23) ribosomal genes should be among the most difficult to change. To study codon essentiality in each of these genes, we attempted to remove all instances of the aforementioned 13 codons (hereafter referred to as “forbidden” codons). In addition, we gauged tolerance for large-scale DNA sequence alterations by shuffling all possible codons to synonymous alternatives. Replacement codons were chosen randomly from a weighted distribution, based on their frequencies in all *E. coli* genes (AUG and UGG codons were unchanged because they uniquely encode Met and Trp, respectively). Finally, we changed 1 non-AUG start codon to AUG, separated six gene overlaps, removed one frameshift, and avoided the use of six restriction sites used in gene assembly (supplementary text). Thus, whereas the protein sequence was 100% identical in our designs, the nucleotide sequence was on average only 65.4% identical, and the codon identity was only 4.44% (corresponding to the unchanged AUG and UGG codons) (table S1). Based on these radical design parameters, we did not expect all design elements to be tolerated. Therefore, individually recoding each gene was the most biologically relevant scale on which to assess the effects of recoding without sacrificing the ability to rapidly map design flaws.

We synthesized recoded genes from DNA microchips (24), transcriptionally fused each to a kanamycin resistance gene (*kan^R*) by isothermal assembly (25), and replaced the corresponding natural gene (one gene per strain) in vivo using λ Red recombination (26) (Fig. 1B). We also introduced *kan^R* downstream of the natural genes and found that 41 of 42 (table S2) allowed insertion with an average growth defect of 15% in LB-Lennox medium (12% in Teknova Hi-Def Azure medium). Insertion downstream of *rplO* was unsuccessful, indicating that disrupting operon structure—and, by extension, refactoring overlapping genes—is a potential failure mode for redesigning genomes. For the recoded genes, we found that 26 of 42 (table S2) were successful, with an average growth defect of 20% in LB-Lennox (14% in Azure) compared with *kan^R* insertion controls (Fig. 2). In the recoded *prfB* strain, removing the frameshift and recoding an upstream AGG codon that may be involved in pausing translation and enhancing frameshifting (15) did not significantly affect fitness (*t* test, *P* = 0.86). Finally, to test the independence of the growth defects, we inserted a recoded *rplM* or *rpsL* gene transcriptionally fused to spectinomycin resistance into three recoded strains with varying fitness (*rpmC_{syn1}*, *rplE_{syn1}*, and *rplP_{syn1}*). All double-mutant strains exhibited better fitness than predicted assuming that the fitness defects were independent, although this does not rule out potential cumulative effects from combining multiple deleterious designs (fig. S1).

The 16 unsuccessfully recoded genes provided an opportunity to identify failure modes for recoding. We coarsely mapped deleterious alleles in the remaining genes by recoding only the C-terminal half of each gene (successful for 9 of 16 genes, table S2). Of these 9 genes, 7 were also amenable to recoding all but the first 30 codons (Fig. 1B).

Although not conclusive based on the limited sample size, these remaining failed replacements may be caused by the disruption of endogenous control mechanisms upstream of the gene (23) or by codon bias affecting expression (7, 12). Using the above synthetic complementation approaches, we recoded a total of 294 of 405 forbidden codons in 35 of 42 targeted essential genes across 35 strains (one recoded gene per strain) (tables S2 and S3). This generated 4375 out of 6496 total desired nucleotide changes and introduced 29 synthesis errors and/or spontaneous mutations (1 error per 436 base pairs) (Fig. 3 and table S4). Although synthesis errors sometimes introduced de novo forbidden codons, additional screening invariably found alternative clones lacking forbidden codons. We hypothesize that the remaining genes (7 of 16) failed due to perturbations in gene expression arising from separating overlapping genes and/or nonviable changes introduced while shuffling codons that were not forbidden.

To determine whether any remaining instances of the forbidden codons were essential, we used coselection multiplex automated genome engineering (CoS-MAGE) (27) to remove all remaining forbidden codons in small groups across a population of cells (111 desired mutations in 45 clones) (Fig. 3 and table S5). The CoS-MAGE recombinants exhibited robust fitness (Fig. 2), indicating that none of the forbidden codons provide a systematic barrier to removal. Furthermore, this suggests that unsuccessful gene replacements using fully recoded cassettes were not due to the removal of forbidden codons. Our initial designs yielded all desired mutations except for one (*rplQ* U162G). Unexpectedly, when we attempted to replace this CUU (Leu) codon using a pool of oligos encoding all Leu, Ile, Val, and Ala codons (table S6), only CUG (Leu), UUG (Leu), and GUG (Val) were not observed (table S7). Therefore,

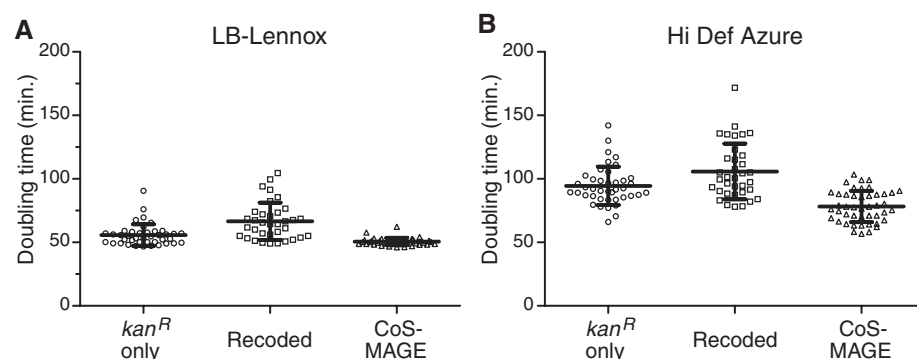


Fig. 2. Recoded strain doubling times. Recoded strain doubling times in (A) LB-Lennox media and (B) Teknova Hi-Def Azure media. Each data point represents the average doubling time of a given strain with a portion of a ribosomal gene recoded (*n* = 3). Error bars for each group represent mean $\pm$ SD. Under assay conditions, the parental strain [*E. coli* MG1655 Δ (*yhbB-bioAB*):[λ cl857 *N*(*cro-ea59*):*tetR-bla*] Δ *mutS::cat*, “EcNR2”) exhibited a 49 ± 4 min doubling time in LB-Lennox and a 84 ± 5 min doubling time in Teknova Hi-Def Azure. Strain genotypes and doubling times are summarized in tables S4 and S5. *Kan^R* insertion into natural sequences (with no recoding) seldom impaired fitness. Still, we could not introduce *kan^R* downstream of *rplO* after three attempts. Fully or partially recoded gene recombinants exhibited the broadest range of fitness defects. For successful recombinants, position of the recoded gene in its operon did not appear to correlate strongly with fitness. The CoS-MAGE recombinants exhibited robust fitness, indicating that all tested forbidden codons are readily dispensable in small groups.

CUU is not essential, but 3 out of 12 tested replacement codons (all ending in UG) were either deleterious or recalcitrant to λ Red-mediated allele replacement in a way that was not anticipated. We note that the native gene sequence at this locus (ACT CTT GCC) contains most of the CTWGG Vsr recognition motif [Vsr is a mismatch repair endonuclease that is somewhat MutS-independent (28)] but that the position (nucleotide 3 instead of nucleotide 2) and identity (T:C instead of T:G) of the oligo-mediated mismatch are noncanonical. We mutated codons 23 and 24 of *vsr* to in-frame stop codons but were still unable to isolate *rplQ* recombinants with CUG, UUG, or GUG codons at position 162, thus suggesting that Vsr is not the cause of these failed replacements. It is likely that further recoding will uncover additional cryptic

design flaws; nevertheless, our strategy is well suited to rapidly identify alternative solutions that are viable.

Our results provide three important insights for designing recoded genomes. First, when tested individually or in groups, all 405 instances of the forbidden codons were nonessential, suggesting that they are amenable to genome-wide removal. Second, our inability to replace CUU with CUG, UUG, and GUG at position 162 in *rplQ* demonstrates that synonymous codons can be non-equivalent in unpredictable ways. Nevertheless, our ability to successfully remove all instances of 13 codons from a panel of highly expressed essential genes indicates that radical genome recoding is feasible. Finally, most of the recoded genes displayed reduced fitness, and combining the cur-

rent designs into a single genome could lead to unacceptable fitness impairment. In contrast, we did not observe significantly altered growth rates for the CoS-MAGE strains in which only forbidden codons were changed (table S5). Therefore, our future strategies for genome-wide codon reassignment will only change codons of interest while selecting for variants with normal growth. This approach leverages diversity and evolution to overcome such uncharacterized genome design constraints, allowing researchers to focus on creating genomes possessing new and useful functions.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Fig. S1

Tables S1 to S11

References (31–36)

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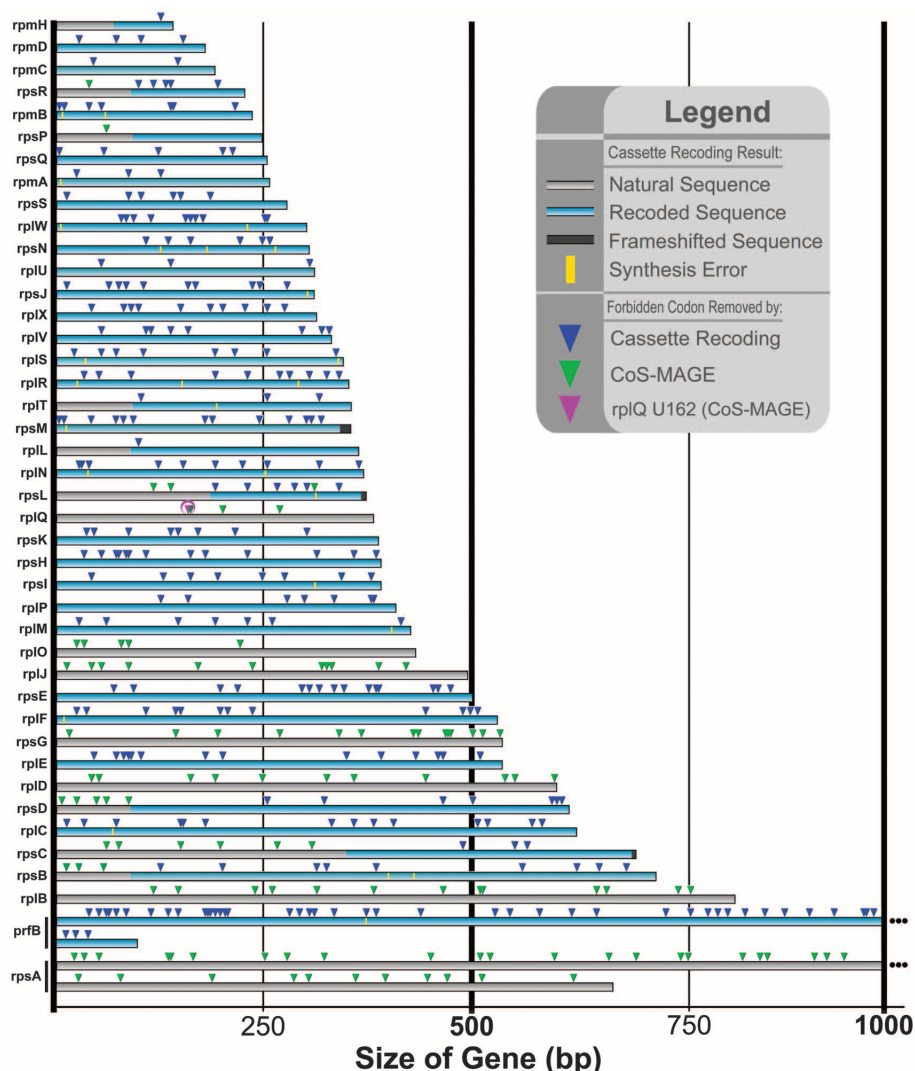


Fig. 3. Schematics of all changes introduced in recoded essential genes. Light gray represents natural DNA sequence, light blue represents recoded sequence (average nucleotide identity = 65.4%), and dark gray represents frameshifted sequences caused by point deletions. Yellow lines indicate missense mutations introduced by gene synthesis errors, none of which introduced forbidden codons. Triangles indicate forbidden codons recoded by gene replacement (blue) or CoS-MAGE (green). The purple triangle in *rplQ* indicates the CUU codon that could not be converted to CUG as originally designed. We exhaustively tested all possible replacement Leu, Ile, Val, and Ala codons, and only CUG, UUG, and GUG were not observed. All 405 instances of the forbidden codons were successfully replaced across 80 strains.

Rapid Adaptation to Climate Facilitates Range Expansion of an Invasive Plant

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Adaptation to climate, evolving over contemporary time scales, could facilitate rapid range expansion across environmental gradients. Here, we examine local adaptation along a climatic gradient in the North American invasive plant *Lythrum salicaria*. We show that the evolution of earlier flowering is adaptive at the northern invasion front where it increases fitness as much as, or more than, the effects of enemy release and the evolution of increased competitive ability. However, early flowering decreases investment in vegetative growth, which reduces fitness by a factor of 3 in southern environments where the North American invasion commenced. Our results demonstrate that local adaptation can evolve quickly during range expansion, overcoming environmental constraints on propagule production.

The contribution of rapid evolution to range expansion and invasion into novel climatic regimes is poorly understood. Several common garden studies of introduced species have demonstrated the reestablishment of latitudinal or altitudinal clines in life-history traits similar to those observed in the native range, im-

plicating a role for adaptive evolution in response to local climate (1–3). However, linking geographical clines in candidate traits to range expansion requires additional lines of evidence. First, fitness trade-offs among life-history traits should be investigated to distinguish adaptive evolution from nonadaptive responses to selection on cor-

related traits (4, 5). Second, reciprocal transplants across environmental gradients must demonstrate crossing reaction norms—the signature of local adaptation—in which locally adapted “home” populations have higher fitness than the non-adapted “away” populations at each site. Third, measurements of natural selection on candidate traits are needed to identify ecologically divergent selection—a geographically shifting optimum—responsible for adaptive clines. Finally, the fitness effect of local adaptation should be compared with other factors thought to facilitate range expansion. We used each of these criteria to link adaptive evolution to the spread of an invasive wetland plant.

We measured natural selection and tested for local adaptation in introduced populations of *Lythrum salicaria* (purple loosestrife—Lythraceae) from eastern North America. This species is one of the world’s most serious wetland invaders and

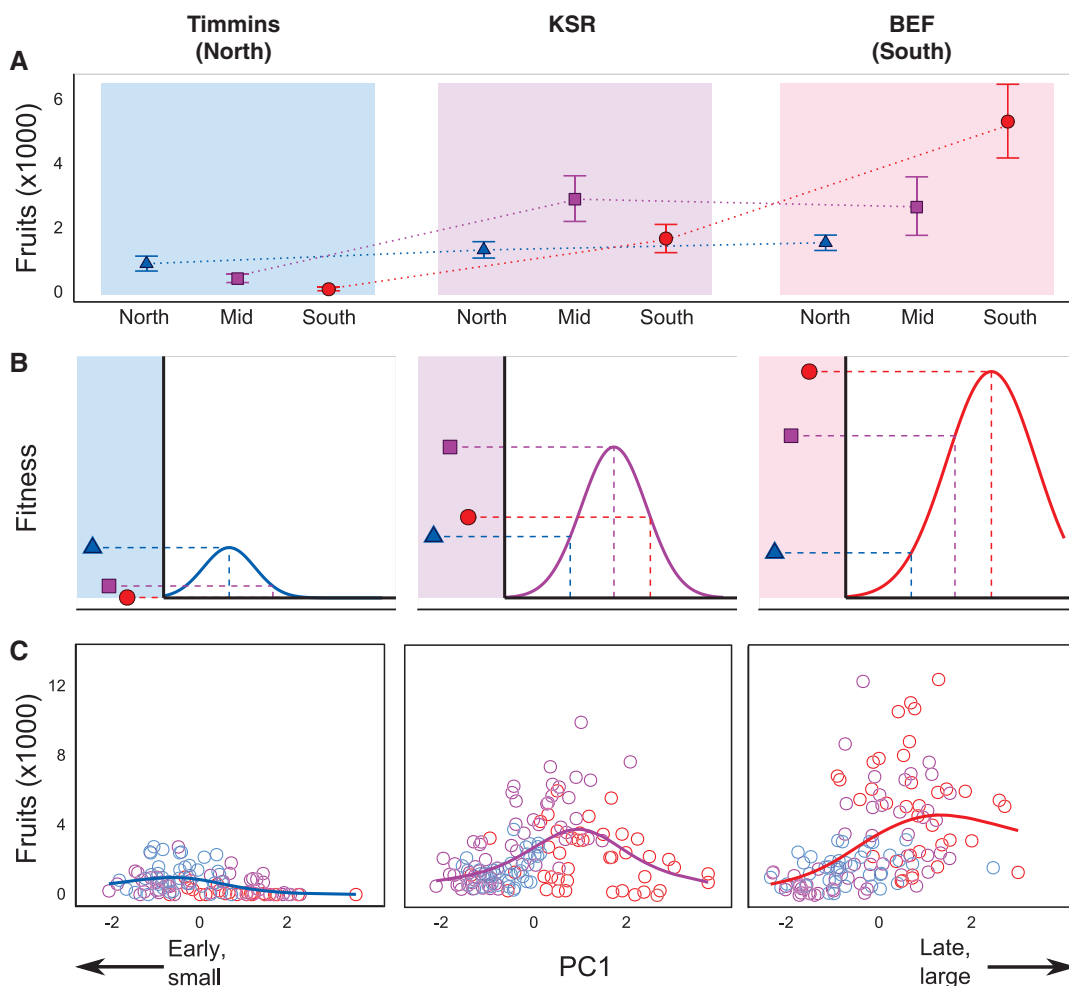
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Fig. 1. Rapid evolution of local adaptation to climate among invasive populations of *L. salicaria* resulting from a latitudinal shift in the adaptive landscape. (A) Evidence for local adaptation based on survival and fruit production data over 4 years (2007–2010) at each of three sites spanning a latitudinal gradient of 10° (~1000 km). Each point is the mean of a pair of populations [$\pm 95\%$ confidence interval (CI)] grown from seeds collected from northern (blue triangles), southern (red circles), and mid-latitude sites (purple squares).

(B) Adaptive landscapes modeled for a single quantitative trait representing a trade-off between reproductive timing versus size at maturity (PC1, x axis) in three environments: short (Timmins), long (BEF), and intermediate growing seasons (KSR). Solid curves in each graph show the predicted adaptive landscape, and dotted lines show the consequences for fitness of populations collected from northern (blue triangles), southern (red circles), and intermediate latitudes (purple squares). (C) Observed cumulative reproductive output (circles) over 4 years (2007–2010) and nonlinear fitness splines (solid curves) of 450 plants from three pairs of populations collected from northern (blue), southern (red), and intermediate latitudes (purple), reciprocally transplanted into the three common garden sites.



is the target of numerous control programs (6, 7). Previous studies have shown the reestablishment of latitudinal clines in growth and phenology in this region (8, 9), with northern populations flowering an average of ~20 days earlier, but at half the size of southern populations, when both are grown in a common environment (4, 5, 9, 10). To determine whether these genetic differences in flowering time and size are locally adaptive, we transplanted seedlings in 2007 from three pairs of populations originating from northern, mid-latitude, and southern populations into each of three common garden sites with contrasting growing seasons and spanning most of the introduced range in eastern North America [Timmins, 48.47°N; Koffler Scientific Reserve (KSR), 44.03°N; Blandy Experimental Farm (BEF), 39.06°N] (11).

Measurements of survival and fruit production over four field seasons (2007–2011) showed the characteristic “home site advantage” predicted by the local adaptation hypothesis—on average, populations from latitudes closest to each garden location had higher fitness than populations from more distant latitudes (Fig. 1A). Local adaptation is frequently observed in native plant populations (12, 13), but in this case historical evidence indicates a relatively recent (<100 years) northward migration of *L. salicaria* into Ontario, Canada, following multiple introductions from Europe to the eastern seaboard of the United States (6). Evidence from neutral markers (14, 15), historical records (6), and patterns of quantitative genetic variation (4) do not support parallel introductions of preadapted populations from Europe to areas of similar climate in North America (11).

Rather, adaptive latitudinal clines in *L. salicaria* have evolved rapidly. We tested for divergent selection among populations responsible for the rapid evolution of local adaptation.

We modeled the adaptive landscape for each point along a latitudinal transect as a Gaussian function defined by three parameters: (i) the phenotypic optimum, (ii) the strength of stabilizing selection, and (iii) the mean fitness at the optimum phenotype. Previous studies showed that natural selection at the mid-latitude site (KSR) favored both early flowering and larger size, but early-flowering plants were constrained to be small, resulting in stabilizing selection along the primary axis of genetic covariance for these traits (PC1) (4, 10). Parameter values for each of our three study sites were inferred from latitudinal clines observed in both common garden (4) and field (9) studies of 20 populations spanning our experimental garden sites (11). Evolution of adaptive clines through ecologically divergent selection is predicted in the parameterized model, because the phenotypic optimum shifts from flowering early at a small size, to flowering later at a large size, as the length of the growing season increases and relaxes selection for early flowering (Fig. 1B). We tested this model by measuring natural selection at each of our common garden locations.

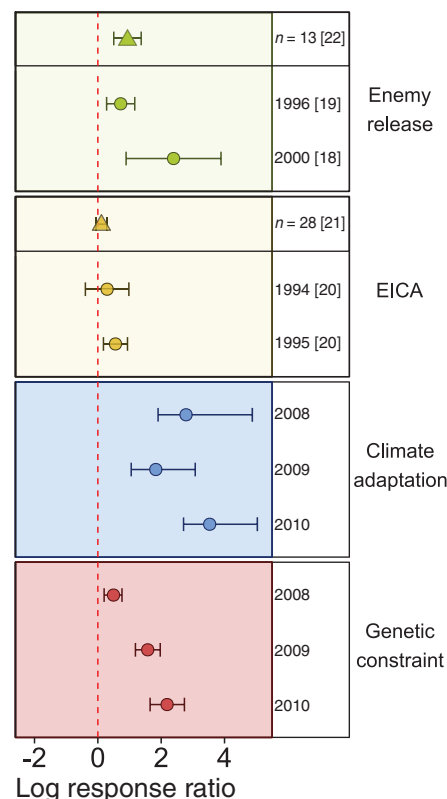
Selection analysis confirmed a geographical shift in the adaptive landscape, from an optimum of early flowering at a small size in the northern common garden to later flowering at a larger size in the south (Fig. 1C). This difference persisted after controlling for region of origin in the selection analysis, ruling out among-population

genetic correlations with other traits, such as frost tolerance or growth rate (11). We cannot rule out within-population genetic correlations with unmeasured traits, but the specific signature of local adaptation provides additional evidence for ecologically divergent selection on PC1 per se. As predicted by the parameterized model, transplanting northern populations to sites with progressively longer growing seasons had little effect on reproductive fitness because fruit production is limited by size in these small plants, resulting in a relatively flat reaction norm (compare blue triangles in Fig. 1, A and B). In contrast, large southern plants acquired ample resources, but their delayed reproduction sharply reduced fitness when transplanted into sites with shorter growing seasons, as predicted by the model (compare red circles in Fig. 1, A and B). Thus, divergent selection favored earlier reproduction in the north but larger size in the south, resulting in an adaptive latitudinal cline in flowering time and size.

Given that clines in eastern North America have established quickly as a result of local adaptation, what are the ecological consequences of rapid adaptation to climate compared with other proposed selective factors? *Lythrum salicaria* has played an important role in the development of two key hypotheses in invasion biology: the enemy release hypothesis (ERH) (16) and the evolution of increased competitive ability (EICA) (17). Escape from two specialist herbivores that dramatically reduce seed production is thought to have facilitated the North American invasion of *L. salicaria*, consistent with the ERH (18, 19). The EICA hypothesis additionally predicts relaxed selection for costly defensive traits, allowing reallocation of resources to growth and reproduction (17). Consistent with this idea, introduced populations of *L. salicaria* have higher reproductive output relative to native conspecifics when grown in a common environment (20). We compare these fitness effects (11) and two recent meta-analyses (21, 22) with our reciprocal transplant results to assess the ecological consequences of local adaptation to climate among invasive populations.

Rapid evolution of earlier flowering is adaptive under short growing seasons (Fig. 1, Timmins site) and increased fitness of northern versus southern populations by as much as 37 times at the northern site (183 versus 5 fruits in 2010) (Fig. 2, Climate adaptation). However, local adaptation of northern populations comes at a cost of reduced vegetative growth, which is maladaptive under longer growing seasons (Fig. 1, BEF site) and reduced reproductive fitness by as much as 90% at the southern site (1433 versus 161 fruits in 2010) (Fig. 2, Genetic constraint). In most years of our study, the magnitude of these fitness effects were significantly higher than the putative fitness increase inferred from EICA studies, and as high as or higher than effects of specialist herbivores (Fig. 2). This indicates that both climate adaptation and genetic constraint strongly affect survival and propagule production in *L. salicaria* populations.

Fig. 2. Comparison of fitness effects (log-response ratio $\pm$ 95% CI) among potential drivers of invasive spread in two meta-analyses of introduced plants (triangles), and three studies of the same factors in *L. salicaria* (circles). Number of species or years of study are shown to the right of each study, along with reference numbers in brackets. Effects of specialist herbivores (enemy release) and evolution of increased competitive ability (EICA) measured in previous studies are compared to the fitness benefit of local adaptation to shorter growing seasons near the northern invasion front at the Timmins site (Climate adaptation), and the fitness cost of early reproduction at the BEF site (Genetic constraint), the region where the North American invasion likely commenced.



We have shown that contemporary evolution of local adaptation to climatic conditions strongly influences seed production of a globally important invasive species—as much as or more so than biotic factors such as enemy release or the evolution of increased competitive ability, which have up to now dominated the literature on plant invasions. Local adaptation can evolve rapidly in outbreeding invaders like *L. salicaria* if multiple introductions from diverse native sources (6, 14, 15) contribute substantial standing genetic variation (5). In such cases, higher recombination rates increase the efficiency of natural selection in invasive populations of outcrossing relative to selfing species. Management efforts and comparative studies of native and introduced populations could be improved by explicitly considering that invasive species are not static entities, but can evolve rapidly, with important implications for future spread.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S3
References (23–36)

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The Invasive Chytrid Fungus of Amphibians Paralyzes Lymphocyte Responses

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The chytrid fungus, *Batrachochytrium dendrobatidis*, causes chytridiomycosis and is a major contributor to global amphibian declines. Although amphibians have robust immune defenses, clearance of this pathogen is impaired. Because inhibition of host immunity is a common survival strategy of pathogenic fungi, we hypothesized that *B. dendrobatidis* evades clearance by inhibiting immune functions. We found that *B. dendrobatidis* cells and supernatants impaired lymphocyte proliferation and induced apoptosis; however, fungal recognition and phagocytosis by macrophages and neutrophils was not impaired. Fungal inhibitory factors were resistant to heat, acid, and protease. Their production was absent in zoospores and reduced by nikkomycin Z, suggesting that they may be components of the cell wall. Evasion of host immunity may explain why this pathogen has devastated amphibian populations worldwide.

Although causes of global amphibian declines are complex (1), the chytrid fungus, *Batrachochytrium dendrobatidis* (2, 3), is now recognized as a leading contributor (1, 4).

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Previous studies in *Xenopus laevis* suggest that both innate immune defenses, such as antimicrobial peptides in the mucus, and adaptive immunity contribute to resistance against *B. dendrobatidis* infection (5). However, lack of extensive lymphocyte infiltration in diseased skin (2, 6) suggests an impaired immune response (7–9). Because evasion of host immunity is a common strategy employed by pathogenic fungi (10, 11), we hypothesized that *B. dendrobatidis* avoids clearance by inhibiting critical immune functions. To test this hypothesis, we examined the effects of *B. dendrobatidis* on peritoneal leukocytes enriched for macrophages, and we cultured *X. laevis* splenocytes stimulated with T lymphocyte-specific

activators (12) or heat-killed bacteria to stimulate B lymphocytes (13) in the presence of *B. dendrobatidis* [see materials and methods in supplementary materials (14)]. Viability and functions of peritoneal phagocytes were not impaired by *B. dendrobatidis* (figs. S1 and S2). However, when splenic lymphocytes were cultured with either live or heat-killed *B. dendrobatidis*, T cell proliferation was reduced (Fig. 1, A and B, and fig. S3, C and E). Live *B. dendrobatidis* cells also inhibited B cell proliferation (fig. S3A). When lymphocytes were separated from *B. dendrobatidis* by a cell-impermeable membrane in a transwell culture system, the fungal cells inhibited lymphocyte proliferation, but less effectively than in coculture (Fig. 1C). The inhibitory effects of *B. dendrobatidis* were replicated in *X. laevis* T and B cell populations enriched by magnetic sorting (fig. S4). Inhibition of T and B lymphocyte proliferation by *B. dendrobatidis* was also observed when the splenocytes were isolated from another frog, *Rana pipiens* (fig. S5). Induced T and B cell proliferation was inhibited in a dose-dependent manner by 24-hour supernatants derived from *B. dendrobatidis* incubated in water (Fig. 1D and fig. S3, B, D, and F). Proliferation of mouse and human lymphocytes was also inhibited by *B. dendrobatidis* supernatants (fig. S6). Frog splenocytes pretreated with *B. dendrobatidis* supernatants for 48 hours had reduced proliferative capacity in response to phytohemagglutinin (PHA), and delayed addition of supernatants at 24 hours after PHA stimulation still inhibited proliferation (fig. S7). Thus, *B. dendrobatidis* can prevent activation and interfere with proliferation after lymphocyte activation has been induced. Further, *B. dendrobatidis* supernatants derived from killed cells inhibited proliferation

(fig. S8). Cells and supernatants from the closely related nonpathogenic chytrid, *Homolaphlyctis polyrhiza* (15), inhibited splenocytes poorly in comparison with those from *B. dendrobatidis*

(fig. S9). Proliferation of mammalian epithelial cell lines [HeLa and Chinese hamster ovary (CHO)] was also inhibited by *B. dendrobatidis* supernatant (fig. S10). These data suggest that

B. dendrobatidis releases a soluble factor that prevents lymphocytes and other cell types from proliferating and renders lymphocytes incapable of normal functions. Thus, immune paralysis is not due to defects in the initial innate immune response of macrophages and neutrophils. Instead, it is due to defects in the lymphocyte-mediated effector arm of the response.

Pathogenic fungi, including *Cryptococcus neoformans* (16), *Aspergillus fumigatus* (17), and *Paracoccidioides brasiliensis* (18), inhibit immune defenses by activating apoptosis signaling pathways. Some fungal products directly induce lymphocyte apoptosis (19). Thus, we investigated whether *B. dendrobatidis* induces splenocyte apoptosis. Resting *X. laevis* splenocytes were cultured across a cell-impermeable membrane from live *B. dendrobatidis* cells in transwells and analyzed for apoptosis by flow cytometry with propidium iodide and annexin V staining (20). Splenocytes exposed to *B. dendrobatidis* in transwell cultures showed significantly increased percentages of apoptotic cells at 48 and 72 hours in comparison with controls (Fig. 2A and fig. S11A). When splenocytes were gated to distinguish T and B cells, apoptosis appeared to be preferentially induced in T cells, but some B cells were also apoptotic (fig. S11B). Concentrated *B. dendrobatidis* supernatants also induced splenocyte apoptosis at 48 hours (Fig. 2B and fig. S12), which was significantly decreased by the pan-caspase inhibitor Z-VAD-FMK (Fig. 2C and fig. S13A) but not by necrostatin-1, an inhibitor of programmed necrosis (21) (fig. S13, B and C). The *B. dendrobatidis* supernatants appeared to activate both the intrinsic and extrinsic

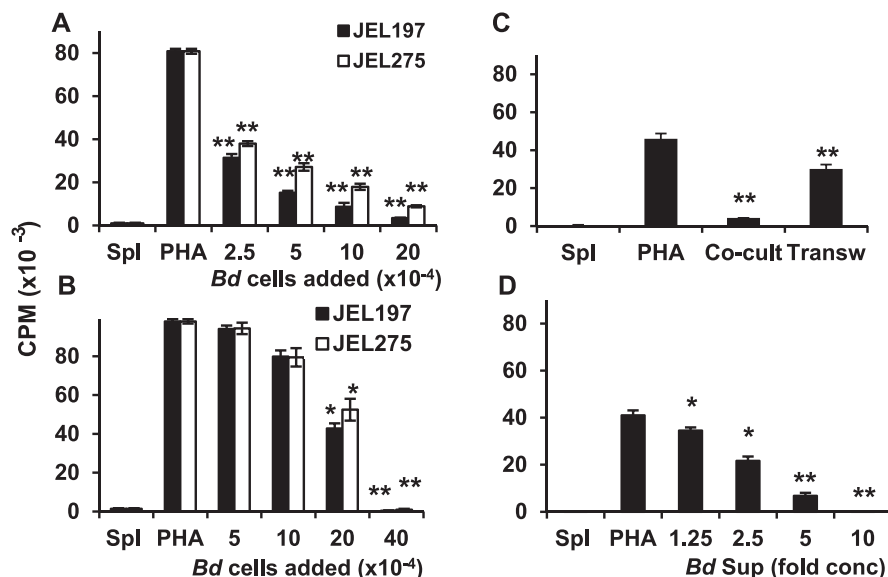


Fig. 1. Inhibition of lymphocyte proliferation by *B. dendrobatidis* (*Bd*). Splenocytes (Spl) from *X. laevis* were cultured alone or with phytohemagglutinin (PHA). PHA-stimulated Spl were cultured alone or with increasing numbers of live (A) or heat-killed (B) *Bd* cells from two pathogenic isolates, JEL197 or JEL275. (C) Spl were cultured as in (A), and PHA-stimulated Spl were cocultured (Co-cult) with or separated from live *Bd* cells by a 0.4-μm pore filter in transwell (Transw). (D) Lymphocytes were cultured as in (A) except that live *Bd* cells were replaced by *Bd* supernatants (Sup) at increasing concentrations. Significantly reduced ³H-thymidine uptake detected as counts per minute (CPM) using a scintillation counter compared to the control treatment, **P* < 0.05, ***P* < 0.01 [analysis of variance (ANOVA) with post hoc test]. CPM data in each panel are means ± SEM of five or more replicate wells and represent three or more similar experiments. Unless noted, JEL197 was the *Bd* isolate used for all cell culture and supernatant experiments.

Fig. 2. Lymphocyte apoptosis induced by *B. dendrobatidis* (*Bd*). Mean percent apoptosis ± SEM of splenocytes (A) cultured with or without *Bd* cells separated by a 0.4-μm filter (20:1 *Bd* to splenocytes) in transwell (three experiments); (B) cultured with or without *Bd* supernatant (Sup) (four experiments); and (C) cultured for 48 hours with or without *Bd* Sup and with or without Z-VAD-FMK (five experiments) and quantified by flow cytometry. (D to F) Caspase activity assays for caspase-3 and -7 (D), caspase-8 (E), and caspase-9 (F) induced by *Bd* Sup, anti-Fas (α-Fas) monoclonal antibody, or corticosterone (Cort) (representative of four experiments). For (A) and (B), percent apoptosis in the presence of *Bd* cells or Sup was significantly greater than that observed for splenocytes alone by a paired Student's *t* test; **P* < 0.05, ***P* < 0.01. For (C), percent apoptosis induced by *Bd* Sup alone was significantly greater than that for splenocytes with no *Bd* Sup or splenocytes treated with both *Bd* Sup and Z-VAD-FMK by a paired Student's *t* test; **P* < 0.05. For (D) to (F), Sup treatments induced significantly greater caspase activity than that of splenocytes alone and of positive controls; ***P* < 0.01 (ANOVA with post hoc test).

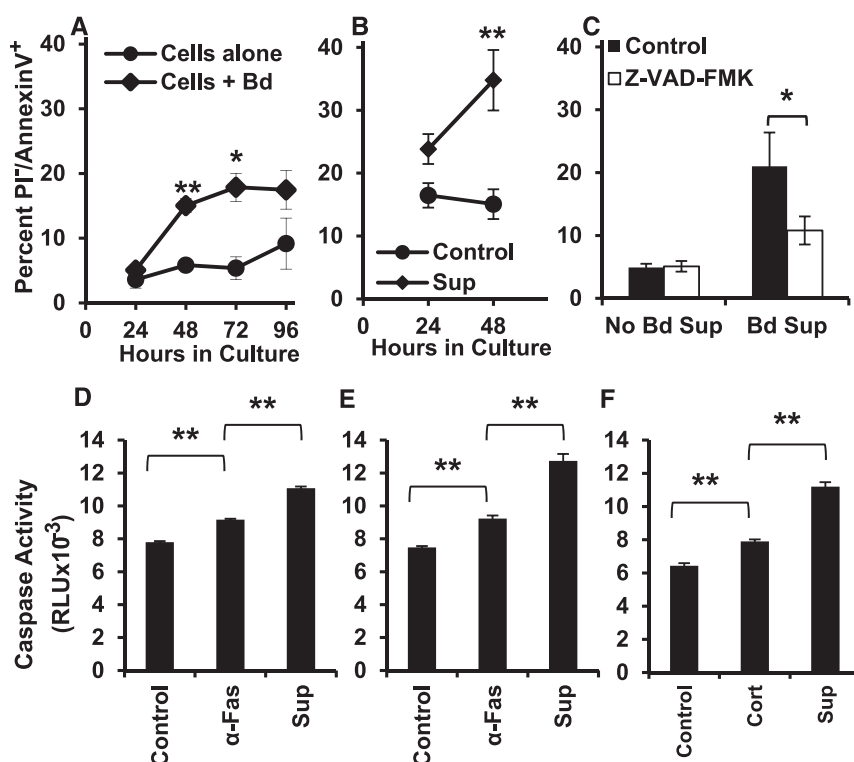
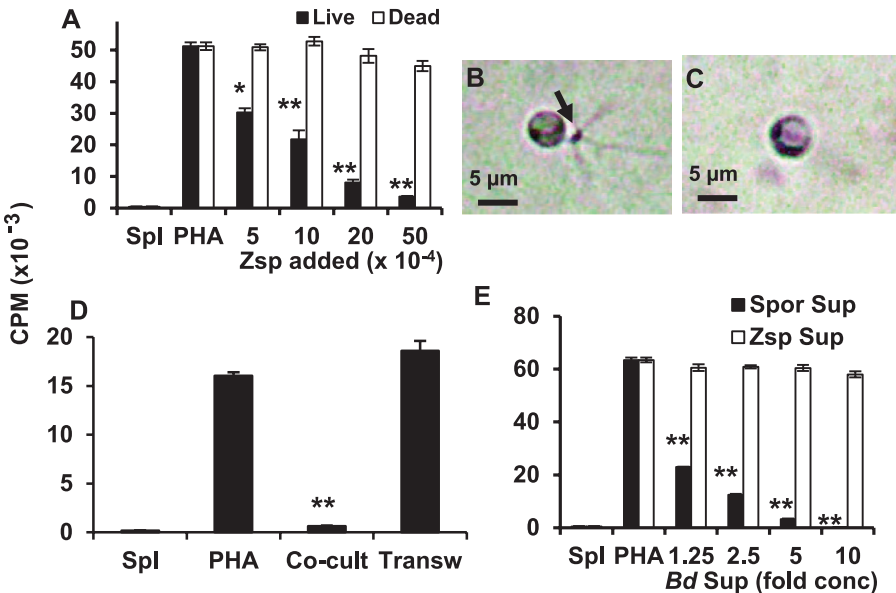


Fig. 3. Effects of *B. dendrobatidis* (Bd) zoospores (Zsp) or zoospore supernatants on lymphocyte proliferation. (A) Splenocytes (Spl) were cultured alone or with PHA. PHA-stimulated Spl were incubated with increasing numbers of live or heat-killed *Bd* Zsp. Live Zsp (B) developed into germlings during the 3-day culture as shown by the formation of rhizoids (arrow); dead Zsp (C) failed to develop (bar: 5 μ m). (D) Spl were cultured as in (A), and PHA-stimulated Spl were cocultured (Co-cult) with or separated from live Zsp by a 0.4- μ m filter in transwell (Transw). (E) Supernatant from whole *Bd* cultures containing both zoospores and sporangia (Spor Sup) or from zoospores alone (Zsp Sup) were concentrated and incubated with PHA-stimulated Spl. Significantly reduced 3 H-thymidine uptake compared to the control treatment, **P* < 0.05, ***P* < 0.01 (ANOVA with post hoc test). CPM in each panel are means $\pm$ SEM of five or more replicate wells and represent three similar experiments.



caspase signaling pathways in splenocytes, as shown by increased activity of caspase-3 and -7 (Fig. 2D), caspase-8 (Fig. 2E), and caspase-9 (Fig. 2F). These results indicate that *B. dendrobatidis* inhibits splenocyte function by activating apoptotic signaling pathways.

B. dendrobatidis has two discernible life stages: the zoospore, which lacks a cell wall, and the mature sporangium (3). To identify the life stages that inhibit lymphocytes, we purified zoospores (22) and mixed them directly with PHA-stimulated splenocytes (Fig. 3A). During 3 days of incubation, zoospores matured (Fig. 3B) and inhibited proliferation (Fig. 3A, solid bars, and fig. S14). Because zoospores can mature rapidly at 26°C, we tested whether heat-killed zoospores (Fig. 3C) also inhibit splenocytes. In contrast to live zoospores or freshly killed mature cells, heat-killed zoospores could not inhibit lymphocyte proliferation (Fig. 3A, open bars). Live zoospores did not inhibit lymphocyte proliferation when physically separated from lymphocytes in a transwell, but reduced proliferation when in direct coculture with lymphocytes (Fig. 3D). Even when zoospores were placed with splenocytes in the top chamber of a transwell, proliferation was not inhibited in the bottom chamber (fig. S14). Furthermore, supernatants from zoospores were not inhibitory (Fig. 3E). These results show that zoospores do not release splenocyte inhibitory factors until they mature.

To biochemically characterize the lymphocyte inhibitory factors released by *B. dendrobatidis*, we subjected concentrated *B. dendrobatidis* supernatants to heat, proteinase K, or acid. The supernatants retained the capacity to inhibit splenocyte proliferation after incubation at 100°C for 30 min (Fig. 4A) or treatment with either proteinase K (Fig. 4B) or trifluoroacetic acid (fig. S15). Proteinase K digested the protein present in the supernatant (fig. S15A), yet the supernatants retained inhibitory activity (Fig. 4B). These results suggest that the inhibitory factors are not proteins.

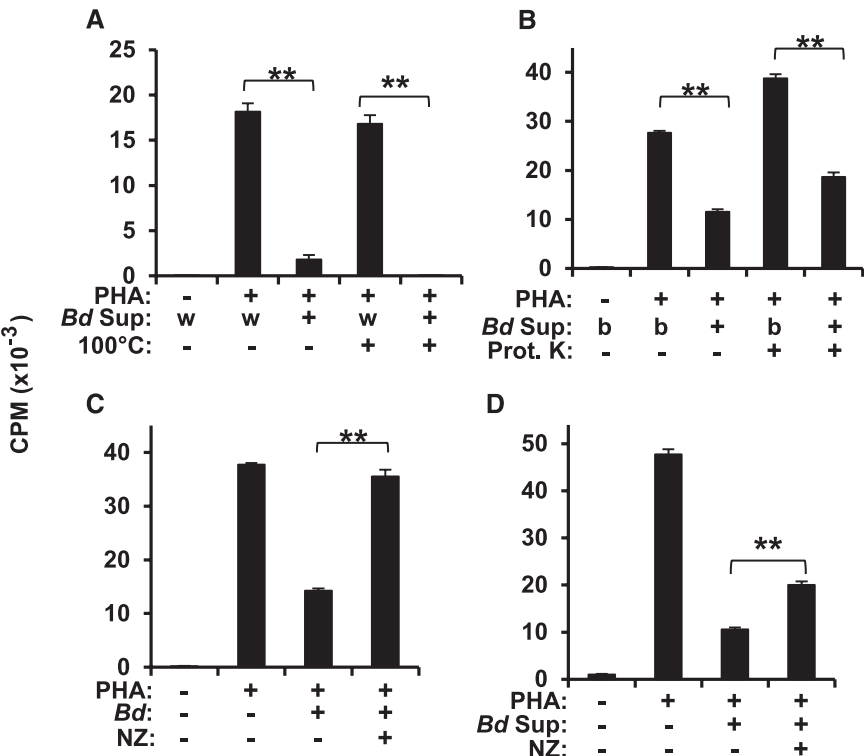


Fig. 4. Partial characterization of *B. dendrobatidis* (Bd) inhibitory factors. (A) Splenocytes were cultured alone or stimulated with PHA. PHA-stimulated splenocytes were exposed to water only (w) or concentrated *Bd* supernatants (Sup) (5 $\times$) that had (+) or had not (–) been incubated at 100°C. (B) Splenocytes were cultured as in (A), and PHA-stimulated splenocytes were incubated with control buffer (b) or with *Bd* Sup (5 $\times$) previously treated with (+) or without (–) proteinase K–conjugated agarose beads (Prot. K). (C and D) Splenocytes were treated as in (A), and PHA-stimulated splenocytes were mixed with (+) or without (–) 10⁵ *Bd* cells (C) or 5 $\times$ *Bd* Sup (D) that had (+) or had not (–) been pretreated with 10 μ g/ml (C) or 5 μ g/ml (D) nikkomycin Z (NZ). For (A) to (D), proliferation was monitored by 3 H-thymidine uptake and CPM were means $\pm$ SEM of five or more replicate wells per treatment. Significant differences between treatments grouped by bars using a Student's *t* test (with correction for multiple tests in the same experiment), ***P* < 0.01. Panels are representative of three similar experiments.

Some inhibitory factors produced by other fungi are cell-wall components (23); therefore, factors produced by *B. dendrobatidis* may also be located in the cell wall. This idea is consistent with the failure of zoospores, which lack a cell wall, to inhibit. To determine whether inhibitory factors are derived from the cell wall, we interfered with cell-wall synthesis using nikkomycin Z (NZ), a chitin synthase inhibitor (24). Preculturing *B. dendrobatidis* with NZ significantly decreased inhibition by both *B. dendrobatidis* cells and supernatants (Figs. 4, C and D). These experiments, along with the observation that non-inhibitory zoospores lack cell walls, suggest that the inhibitory factors produced by *B. dendrobatidis* are cell-wall components. Chitin and β -1,3-glucan are the main structural cell-wall components of many fungi (25). Therefore, we conducted experiments to determine whether they might be inhibitory. Treatment of *B. dendrobatidis* supernatants with β -glucanases and chitinases did not affect the inhibitory activity (fig. S16). Furthermore, treatment of proliferating lymphocytes with a soluble β -glucan (laminarin) did not inhibit function (fig. S16). Thus, the inhibitory factor does not appear to be a β -glucan or chitin.

We conclude that *B. dendrobatidis*, like other pathogenic fungi, produces toxic factors that inhibit potentially protective host immune responses and likely impair the function of other cells in close proximity. Soluble molecules released by *B. dendrobatidis* inhibited proliferation of amphibian and mammalian lymphocytes and induced apoptosis of target cells by activating both intrinsic and extrinsic pathways. The role of phagocytic cells (macrophages and neutrophils) in controlling chytridiomycosis is not yet well understood. These cells can engulf *B. dendrobatidis*, and accessory functions do not appear to be impaired by *B. dendrobatidis* supernatants. Because these soluble mycotoxins inhibited proliferation and caused death of nonlymphoid cell lines, they are more broadly cytotoxic and could be responsible for other symptoms of chytridiomycosis including disruption of the skin (2, 7, 26) and behavioral changes, such as lethargy and loss of righting reflex (6, 7). One or more of the factors produced by *B. dendrobatidis* may be derived from the cell wall. The capacity of *B. dendrobatidis* to evade protective immune responses helps to explain how this fungus can be so lethal to amphibians lacking effective innate defenses (27) and why some amphibian species with more robust innate responses persist with mild infections as *B. dendrobatidis* reservoirs (28–30).

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Supplementary Materials

www.sciencemag.org/content/342/6156/366/suppl/DC1

Materials and Methods

Figs. S1 to S16

References (31–66)

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Measuring Chromatin Interaction Dynamics on the Second Time Scale at Single-Copy Genes

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The chromatin immunoprecipitation (ChIP) assay is widely used to capture interactions between chromatin and regulatory proteins, but it is unknown how stable most native interactions are. Although live-cell imaging suggests short-lived interactions at tandem gene arrays, current methods cannot measure rapid binding dynamics at single-copy genes. We show, by using a modified ChIP assay with subsecond temporal resolution, that the time dependence of formaldehyde cross-linking can be used to extract in vivo on and off rates for site-specific chromatin interactions varying over a ~100-fold dynamic range. By using the method, we show that a regulatory process can shift weakly bound TATA-binding protein to stable promoter interactions, thereby facilitating transcription complex formation. This assay provides an approach for systematic, quantitative analyses of chromatin binding dynamics in vivo.

The chromatin immunoprecipitation (ChIP) assay is an approach for determining where chromatin-binding factors interact with DNA sequences and as such has provided fundamental insight into where and how gene regulatory processes occur in cells. In the ChIP assay, cellular constituents are cross-linked with formaldehyde, the isolated chromatin is fragmented,

and protein-DNA complexes are then recovered by immunoprecipitation using an antibody that detects a chromatin-associated protein of interest. DNA sequences in the immunoprecipitate are then inventoried by polymerase chain reaction. The assay accurately defines where proteins bind (*I*), but it provides limited information about how stable the interactions are. For example, a relatively high ChIP signal could reflect high-occupancy stable binding or that a low-occupancy dynamic interaction was trapped owing to the long formaldehyde incubation period used in standard assays. In fact, live-cell imaging approaches indicate that many chromatin interactions are exceedingly short-lived (2, 3), although such techniques do not provide high-resolution data regarding chromatin binding location. Precise chromatin

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location information can be obtained by competition ChIP, a method that monitors the replacement rate by a differentially tagged factor of interest. However, the time resolution is limited to ~20 min owing to the delay required to generate the competitor species [e.g., (4–6)]. A general assay that provides quantitative measures of site-specific on and off rates is essential for defining chromatin regulatory events as they occur in vivo.

To measure chromatin-binding dynamics in vivo, we developed and applied a mathematical model based on standard principles of chemical kinetics that describes the dependence of ChIP signal on formaldehyde cross-linking time. In this method, which we call cross-linking kinetic (CLK) analysis (7), the mathematical relationship between cross-linking time and ChIP signal is used to extract the overall on rate (the product of the second order rate constant, k_{ab} , and the chromatin binding factor concentration, C_{TF}), the off rate, k_d ; and the fraction of bound chromatin sites at steady state, θ_b^0 . If C_{TF} is known, then the value of k_a can be determined. From k_a , the half-life, $t_{1/2}$, of the chromatin complex can be calculated ($t_{1/2} = \ln 2/k_d$). Figure 1A illustrates the model for a chromatin interaction with a relatively high on rate (left) or low on rate (right). Both complexes have the same off rate, so the higher on rate gives rise to a higher fractional occupancy before addition of formaldehyde ($t = 0$). If formaldehyde cross-linking occurs rapidly as expected (supplementary text), then complexes will be cross-linked at this rapid rate driven by cross-linking kinetics (row labeled $t = 1$ s), fixing the in vivo occupancy in each cell within the first few seconds. At longer formaldehyde incubation times, the unbound chromatin sites become occupied and cross-linked at a rate driven by $k_a C_{TF}$, resulting in an additional increase in the ChIP signal over time. Simulations (Fig. 1B) show this biphasic behavior. The inflection or “knee” in the curves reveals the fractional occupancy in the cell population within the first few seconds of cross-linking. To better constrain the model fits of the data, we made measurements with cells expressing two different concentrations of the transcription factor (TF) of interest and fit the two data sets simultaneously.

To test the CLK method, we analyzed Gal4 binding to the single upstream activation sequence in the *GAL3* promoter. The Gal4 system has provided a paradigm for transcriptional regulation (8), but the in vivo stability of the Gal4-promoter interaction has been the subject of debate (9, 10). A quench-flow apparatus was adapted to acquire formaldehyde-treated samples on the subsecond time scale, and longer time points were obtained by hand mixing before quenching in glycine (supplementary text). As predicted by the simulations (Fig. 1 and figs. S2 and S3), the ChIP signal increased dramatically at short formaldehyde incubation times (<5 s) and then gradually after longer incubation times (Fig. 2A, blue curve). The time dependence of the ChIP signal substantiates several key aspects of the model (fig. S4), and other fundamental suppositions were validated experimentally. First, the steep increase in

ChIP signal at short cross-linking times demonstrates that cross-linking occurred rapidly and that glycine efficiently quenched the reaction (Fig. 2, A and C), as stipulated in the model. The curve was shifted upward in cells with a 2.5-fold increase in Gal4 (Fig. 2A, red curve), consistent with the time dependence of the slower phase of the ChIP signal being driven by the overall on rate for Gal4 chromatin binding and not formaldehyde reaction kinetics. In the model, the ChIP assay rapidly captures specifically bound TFs but does not inactivate or nonspecifically cross-link the remaining TF pool. In fact, the Gal4-promoter interaction occurred in cells even when binding was induced after formaldehyde pretreatment (Fig. 2B). Thus, Gal4 was not nonspecifically inactivated by formaldehyde. Moreover, the levels of soluble Gal4 and other proteins were reduced less than twofold in cell extracts after formaldehyde incubation, and their apparent molecular weights were not detectably affected (Fig. 2D and fig. S1). In addition, ChIP signals were indistinguishable over an eightfold range of formaldehyde concentration (Fig. 2E), demonstrating that formaldehyde was not limiting in the reaction. CLK analysis revealed that the Gal4-*GAL3* interaction had a $t_{1/2}$ of about 10 min (Fig. 2A and table S9), suggesting that a single Gal4 complex facilitates multiple rounds of transcription initiation. Combined with the low fractional promoter occupancy (~0.17), we conclude that the *GAL3* gene is likely transcribed in infrequent bursts.

To better define the dynamic range of the CLK method, we analyzed two TFs whose widely divergent dynamic behavior could be independently measured by fluorescence recovery after photobleaching (FRAP). FRAP was possible in these cases because the fluorescently tagged factors interact with tandem arrays of binding sites, making the chromosomal loci visible by microscopy. The

CLK measured $t_{1/2}$ for the interaction of Ace1-green fluorescent protein (GFP) with the *CUP1* gene array (11) was 11 s, in excellent agreement with the value of 31 s obtained by FRAP (Fig. 3, A and B, and table S8). The interaction of LacI-GFP with an array of *Lac* operators (12) was far more stable, and the two methods yielded $t_{1/2}$ values that differ by less than threefold (Fig. 3, C and D, and table S8). Thus, as validated by an independent approach, the CLK method can reveal rank-ordered estimates of TF-chromatin interaction stability over a wide range in vivo, including interactions that persist for mere seconds. Compared with other methods, the CLK method increases the time resolution of chromatin dynamics at single-copy loci by two to three orders of magnitude.

To further explore transcription dynamics using this method, we investigated the interaction of the TATA-binding protein (TBP) with each of seven different promoters possessing diverse transcriptional activities and driven by RNA polymerases (Pols) I, II, or III. Consistent with expectation (13), the Pol III-driven *SNR6* promoter had the highest occupancy; however, occupancies of all promoters were well below saturation (Fig. 4A and supplementary text). Moreover, TBP-promoter interactions varied dramatically, with $t_{1/2}$ values ranging from 1 to about 30 min (Fig. 4B and table S7), and in many cases half-lives were much shorter than distinguishable by any other technique. To test whether the method can quantify a dynamic difference associated with a perturbation in cellular transcription, we compared TBP dynamics in wild-type (WT) and *mot1-42* cells. Mot1 is an essential regulator of TBP, which uses its adenosine triphosphatase activity to dissociate TBP from DNA in vitro (14). Evidence supports a direct role for Mot1 in gene activation, but how it accomplishes this is unknown. By using *URA1* as a model Mot1-activated gene (15), we observed dramatically different CLK

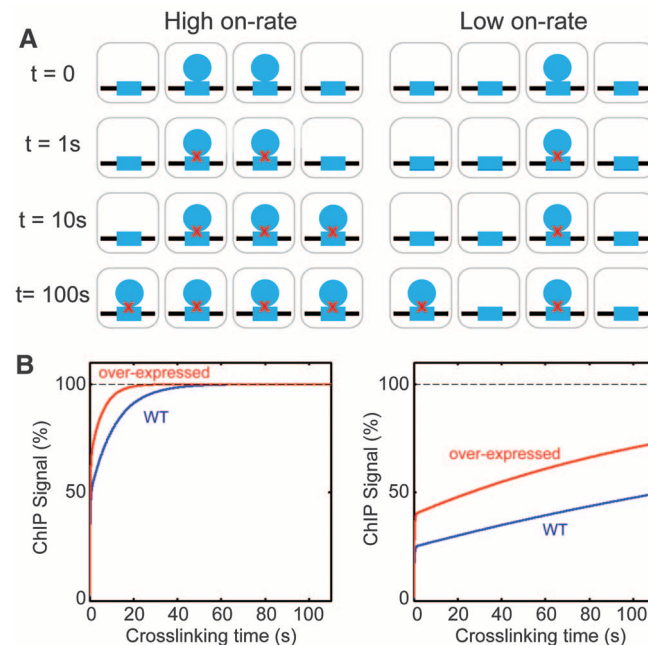


Fig. 1. Overview of the CLK model. (A) Schematic showing a chromatin site (blue rectangle) interacting with a transcription factor (blue circle) in a population of four cells in which chromatin binding has a relatively high (left) or low (right) on rate, but in both cases the off rate is the same. Rows descending from $t = 0$ show how the site occupancy in the cell population is predicted to change after addition of formaldehyde for 1, 10, or 100 s. Red X's indicate cross-linking. (B) Simulations of the two scenarios in (A) using the CLK model (blue lines). The red lines show simulations in which the TF concentration was increased threefold.

Fig. 2. CLK analysis of Gal4 and tests of model assumptions. (A) Model fits of CLK data for Gal4 binding to the *GAL3* promoter in cells with WT Gal4 levels (blue line) and cells with 2.5-fold overexpression of Gal4 (red line). (Inset) The first 5 s of a time course from cells with WT Gal4 levels. Error bars indicate SD. (B) Gal4 ChIP results obtained with cells treated as shown in the schematic. ChIP signals were obtained in formaldehyde-treated, uninduced cells (1), formaldehyde-treated cells subsequently induced by addition of galactose (2), and cells induced with galactose and subsequently treated with formaldehyde (3). Note that Gal4 chromatin binding was fully inducible in formaldehyde-treated cells. (C) Glycine addition before formaldehyde (Form) prevents cross-linking. The graph shows the relative Gal4 ChIP signal obtained when glycine was added before formaldehyde or 8 min after formaldehyde treatment, compared with cells in which no formaldehyde was added. (D) Relative soluble Gal4 protein level in extracts from cells treated with formaldehyde for the indicated times. Gal4 was quantified by Western blotting. (E) Gal4 ChIP signals at *GAL3* obtained by using cells treated with 1 or 8% formaldehyde for the indicated times. ChIP signals did not depend on formaldehyde concentration.

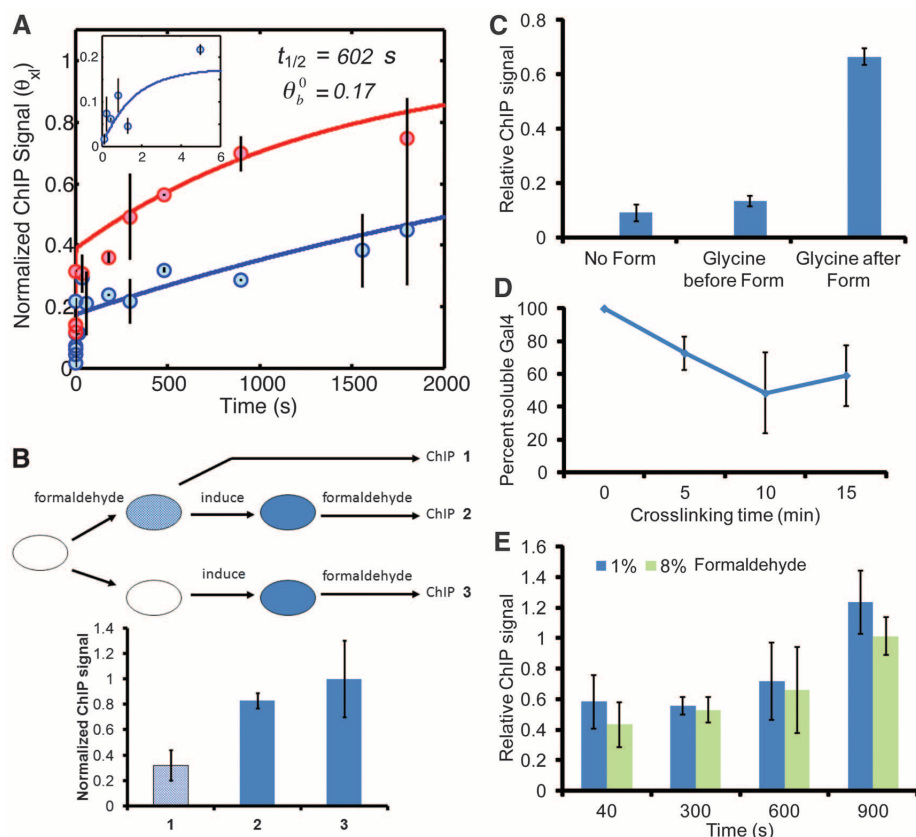
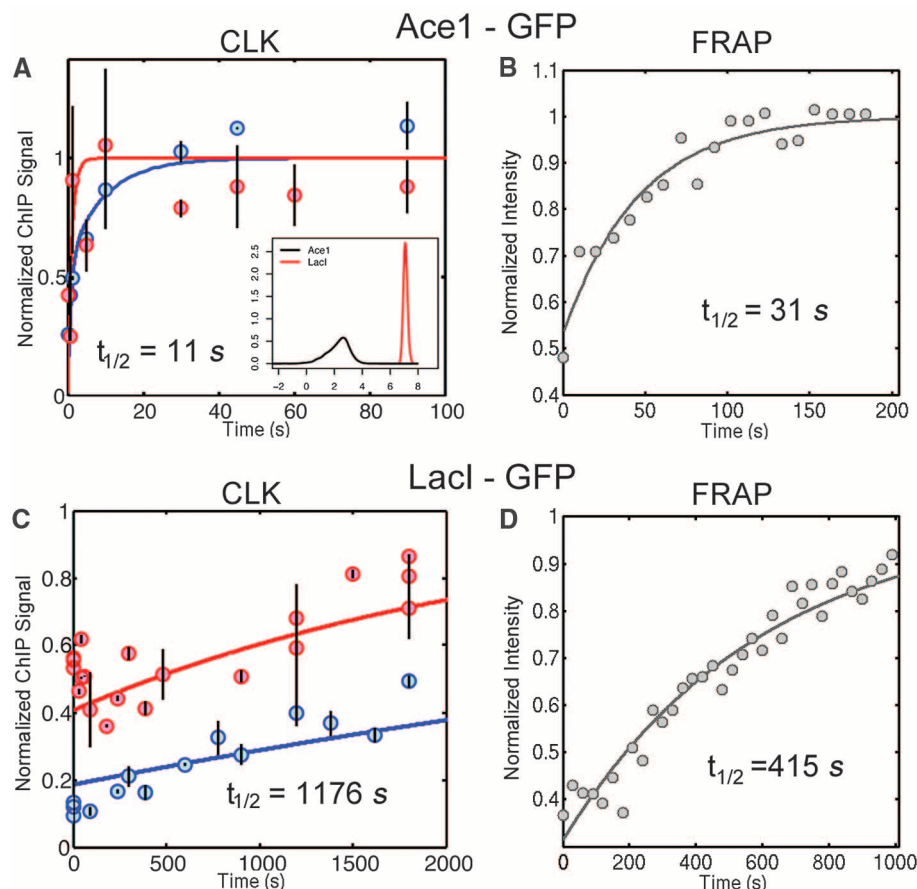


Fig. 3. Comparison of TF-chromatin dynamics by CLK and FRAP. (A) Model fits of CLK data for Ace1-GFP binding to *CUP1* in cells with two different expression levels of Ace1-GFP (low, blue curve; high, red curve; table S2). (Inset) Distributions of $\ln t_{1/2}$ values obtained from multiple independent fits of the Ace1-GFP or LacI-GFP CLK data [shown in (C) and (7)]. (B) FRAP of Ace1-GFP in cells with low Ace1-GFP levels. (C) Model fits of CLK data for LacI-GFP binding to the *Lac* array in cells with low (blue curve) or high (red curve) levels of LacI-GFP (table S2). (D) FRAP of LacI-GFP in cells with low LacI-GFP levels.



curves for TBP binding to the *URA1* promoter in WT and *mot1-42* cells (Fig. 4C and table S7). Biochemical results suggested that Mot1 would activate *URA1* expression by displacing stably bound but inactive TBP from the promoter. However, mutation of Mot1 caused TBP binding to be far more dynamic than in WT cells (Fig. 4D). Similar results were observed at *INO1*, another Mot1-regulated promoter (fig. S13 and table S7).

To reconcile the CLK data with Mot1's biochemical activity and shed light on the process of transcription complex assembly in vivo, we compared the genome-wide TBP ChIP signal at a single cross-linking time in WT and *mot1-42* cells. Mutation of Mot1 increased the TBP ChIP signal at Pol II promoters, and the increase extended well outside the average nucleosome-free promoter region of about 200 base pairs and into flanking transcribed regions (Fig. 4E and fig. S14). TFIIB is a hallmark of transcriptional activity, but in contrast the TFIIB ChIP signal decreased over these same regions. Thus, unstable TBP complexes detectable in *mot1-42* cells were not associated with TFIIB and were transcriptionally inactive. Stable

TBP complexes are apparently better substrates for TFIIB binding, and in turn the binding of TFIIB and other factors can block TBP clearance by Mot1 (14). Rather than catalyzing dissociation of stable TBP interactions, these results reveal that Mot1 is responsible for dissociating weakly bound TBPs at diverse sites, thereby facilitating more stable TBP binding in functional transcription complexes. This enzyme-catalyzed change in TBP dynamics appears essential for proper gene expression; analogous processes may facilitate functional high-affinity chromatin binding at the expense of weak binding by other TFs as well.

The CLK assay yields estimates of physical kinetic parameters as opposed to relative rates, and it is applicable over a much broader time scale than competition ChIP because it is not limited by the time required to synthesize or activate a competitor molecule. This will permit rapid chromatin interaction dynamics for a factor to be compared directly to kinetic parameters for functionally related factors or processes. The CLK methodology is in principle not limited to yeast, and it is based on ChIP, one of the most widely used as-

says in chromatin research. Our data suggest an explanation for why there is no detectable stable chromatin-bound TBP as judged by live-cell imaging (16) but there are stable TBP complexes as judged by competition ChIP (4). The CLK results show that TBP fractional occupancies are low. Thus, although there are stable TBP-promoter complexes in vivo, most promoters are not occupied at steady state. The unexpectedly low occupancies are consistent with results showing that transcription in vivo occurs via uncoordinated stochastic cycles separated in time (17, 18). CLK results also illustrate the danger of inferring relative occupancies or dynamics from ChIP assays using single, long formaldehyde incubation times. TBP ChIP signals are much greater in *mot1-42* cells than in WT cells, but the higher ChIP signals result from highly dynamic TBP molecules being trapped during the formaldehyde incubation period, rather than reflecting stable TBP binding.

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Supplementary Materials

www.sciencemag.org/content/342/6156/369/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S15
Tables S1 to S9
References (19–37)

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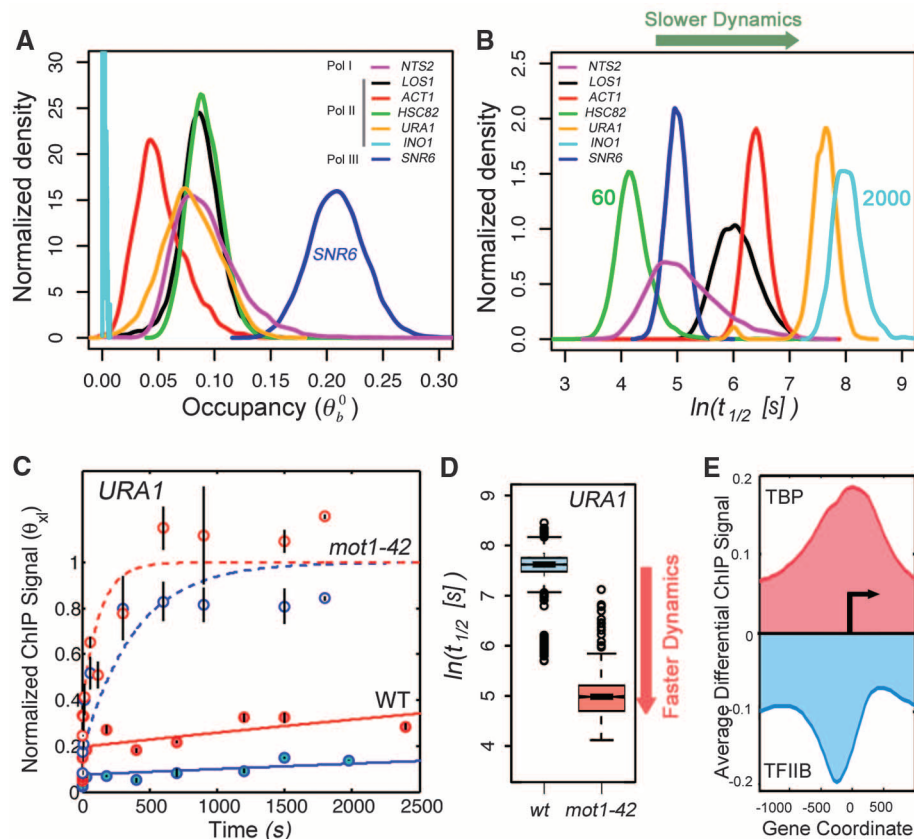


Fig. 4. TBP dynamics and regulation by Mot1. (A) Distributions of TBP occupancy at different yeast promoters obtained by multiple independent fits of the CLK data (7). (B) Distributions of TBP-promoter half-lives (7), whose mean values vary from 60 to about 2000 s. (C) Model fits of CLK data for TBP binding to the Mot1-activated *URA1* promoter in WT (solid lines) and *mot1-42* cells (dashed lines). Data and fits from cells expressing WT levels of TBP are shown in blue; results from cells overexpressing TBP are in red. (D) Box plots for distribution of $t_{1/2}$ values (log scale) for TBP binding to the Mot1-activated *URA1* promoter in WT (blue) and *mot1-42* cells (red). (E) Average genome-wide $\log_2$ differential TBP and TFIIB ChIP-chip signals at promoters in *mot1-42* versus WT cells shown with respect to the transcription start site (arrow) (7).

Sleep Drives Metabolite Clearance from the Adult Brain

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The conservation of sleep across all animal species suggests that sleep serves a vital function. We here report that sleep has a critical function in ensuring metabolic homeostasis. Using real-time assessments of tetramethylammonium diffusion and two-photon imaging in live mice, we show that natural sleep or anesthesia are associated with a 60% increase in the interstitial space, resulting in a striking increase in convective exchange of cerebrospinal fluid with interstitial fluid. In turn, convective fluxes of interstitial fluid increased the rate of β -amyloid clearance during sleep. Thus, the restorative function of sleep may be a consequence of the enhanced removal of potentially neurotoxic waste products that accumulate in the awake central nervous system.

Despite decades of effort, one of the greatest mysteries in biology is why sleep is restorative and, conversely, why lack of sleep impairs brain function (1, 2). Sleep deprivation reduces learning, impairs performance in cognitive tests, prolongs reaction time, and is a common cause of seizures (3, 4). In the most extreme case, continuous sleep deprivation kills rodents and flies within a period of days to weeks (5, 6). In humans, fatal familial or sporadic insomnia is a progressively worsening state of sleeplessness that leads to dementia and death within months or years (7).

Proteins linked to neurodegenerative diseases, including β -amyloid (A β) (8), α -synuclein (9), and tau (10), are present in the interstitial space surrounding cells of the brain. In peripheral tissue, lymph vessels return excess interstitial proteins to the general circulation for degradation in the liver (11). Yet despite its high metabolic rate and the fragility of neurons to toxic waste products, the brain lacks a conventional lymphatic system. Instead, cerebrospinal fluid (CSF) recirculates through the brain, interchanging with interstitial fluid (ISF) and removing interstitial proteins, including A β (12, 13). The convective exchange of CSF and ISF is organized around the cerebral vasculature, with CSF influx around arteries, whereas ISF exits along veins. These pathways were named the glymphatic system on the basis of their dependence on astrocytic aquaporin-4 (AQP4) water channels and the adoption of functions homologous to peripheral lymphatic removal of interstitial metabolic byproducts (14). Deletion of AQP4 channels reduces clearance of exogenous A β by 65%, suggesting that convective movement of ISF is a substantial contributor

to the removal of interstitial waste products and other products of cellular activity (12). The interstitial concentration of A β is higher in awake than in sleeping rodents and humans, possibly indicating that wakefulness is associated with increased A β production (15, 16). We tested the alternative hypothesis that A β clearance is increased during sleep and that the sleep-wake cycle regulates glymphatic clearance.

We used in vivo two-photon imaging to compare CSF influx into the cortex of awake, anesthetized, and sleeping mice. The fluorescent tracers were infused into the subarachnoid CSF via a cannula implanted in the cisterna magna for real-time assessment of CSF tracer movement. Electroencephalography (EEG) and electromyography (EMG) were recorded in order to continuously monitor the state of brain activity (Fig. 1A and fig. S1). In initial experiments, the volume and rate of tracer infusion were adjusted so as to avoid changes in behavior state or EEG (fig. S1). Because mice sleep much of the day, a small molecular weight tracer, fluorescein isothiocyanate (FITC)-dextran (3 kD) in aCSF, was infused at midday (12 to 2 p.m.) via the cannula implanted in the cisterna magna. In sleeping mice, a robust influx of the fluorescent CSF tracer was noted along periarterial spaces, in the subpial regions, and in the brain parenchyma similar to previous findings in anesthetized mice (Fig. 1, B and C, and fig. S2) (12). EEG power spectrum analysis depicted a relatively high power of slow waves that is consistent with sleep (Fig. 1D). CSF tracer infusion (Texas red-dextran, 3 kD) was repeated in the same mouse after it was awakened through gentle handling of its tail. Unexpectedly, arousal sharply reduced tracer influx compared with that of the sleeping state. Periarterial and parenchymal tracer influx was reduced by ~95% in awake as compared with sleeping mice during the 30-min imaging session (Fig. 1, B and C, and fig. S2). EEG showed a reduction in the relative prevalence of slow (delta) waves concomitant with a significant increase in the power of fast activity, confirming that the animals were awake ($n = 6$

mice, $P < 0.05$, paired t test) (Fig. 1D). To investigate whether the state of brain activity indeed controlled CSF influx, we repeated the experiments in a new cohort of mice in which all experiments were performed when the animals were awake (8 to 10 p.m.). Because mice normally do not sleep at this time of day, we first evaluated CSF tracer influx in the awake state by means of intracisternal infusion of FITC-dextran. CSF tracer influx into the brain was largely absent and only slowly gained access to the superficial cortical layers (Fig. 1, E and F, and fig. S2). After 30 min imaging of CSF tracer in the awake state, the animals were anesthetized with intraperitoneal administration of ketamine/xylazine (KX). Texas red-dextran was administered 15 min later, when a stable increase in slow wave activity was noted (Fig. 1, E and F). Texas red-dextran rapidly flushed in along periarterial spaces and entered the brain parenchyma at a rate comparable with that of naturally sleeping mice (Fig. 1, B and E). Ketamine/xylazine anesthesia significantly increased influx of CSF tracer in all mice analyzed [$n = 6$ mice, $P < 0.05$, two-way analysis of variance (ANOVA) with Bonferroni test], which was concomitant with a significant increase in the power of slow wave activity ($n = 6$ mice, $P < 0.05$, paired t test) (Fig. 1, G and F). Thus, glymphatic CSF influx is sharply suppressed in conscious alert mice as compared with naturally sleeping or anesthetized littermates.

Influx of CSF is in part driven by arterial pulse waves that propel the movement of CSF inward along periarterial spaces (12). It is unlikely that diurnal fluctuations in arterial pulsation are responsible for the marked suppression of convective CSF fluxes during wakefulness because arterial blood pressure is higher during physical activity. An alternative possibility is that the awake brain state is linked to a reduction in the volume of the interstitial space because a constricted interstitial space would increase resistance to convective fluid movement and suppress CSF influx. To assess the volume and tortuosity of the interstitial space in awake versus sleeping mice, we used the real-time iontophoretic tetramethylammonium (TMA) method in head-fixed mice (Fig. 2A and fig. S3) (17, 18). TMA recordings in cortex of sleeping mice collected at midday (12 to 2 p.m.) confirmed that the interstitial space volume fraction (α) averaged $23.4 \pm 1.9\%$ ($n = 6$ mice) (19). However, the interstitial volume fraction was only $14.1 \pm 1.8\%$ in awake mice recorded at 8 to 10 p.m. ($n = 4$ mice, $P < 0.01$, t test) (Fig. 2B). Analysis of cortical EEG recorded by the TMA reference electrode confirmed that the power of slow wave activity was higher in sleeping than in awake mice, which is concurrent with a lower power of high-frequency activity (Fig. 2C).

To further validate that the volume of the interstitial space differed in awake versus sleeping mice, we also obtained TMA recordings in awake mice in the late evening (8 to 10 p.m.) and repeated the recordings in the same mice after administration of ketamine/xylazine. This approach,

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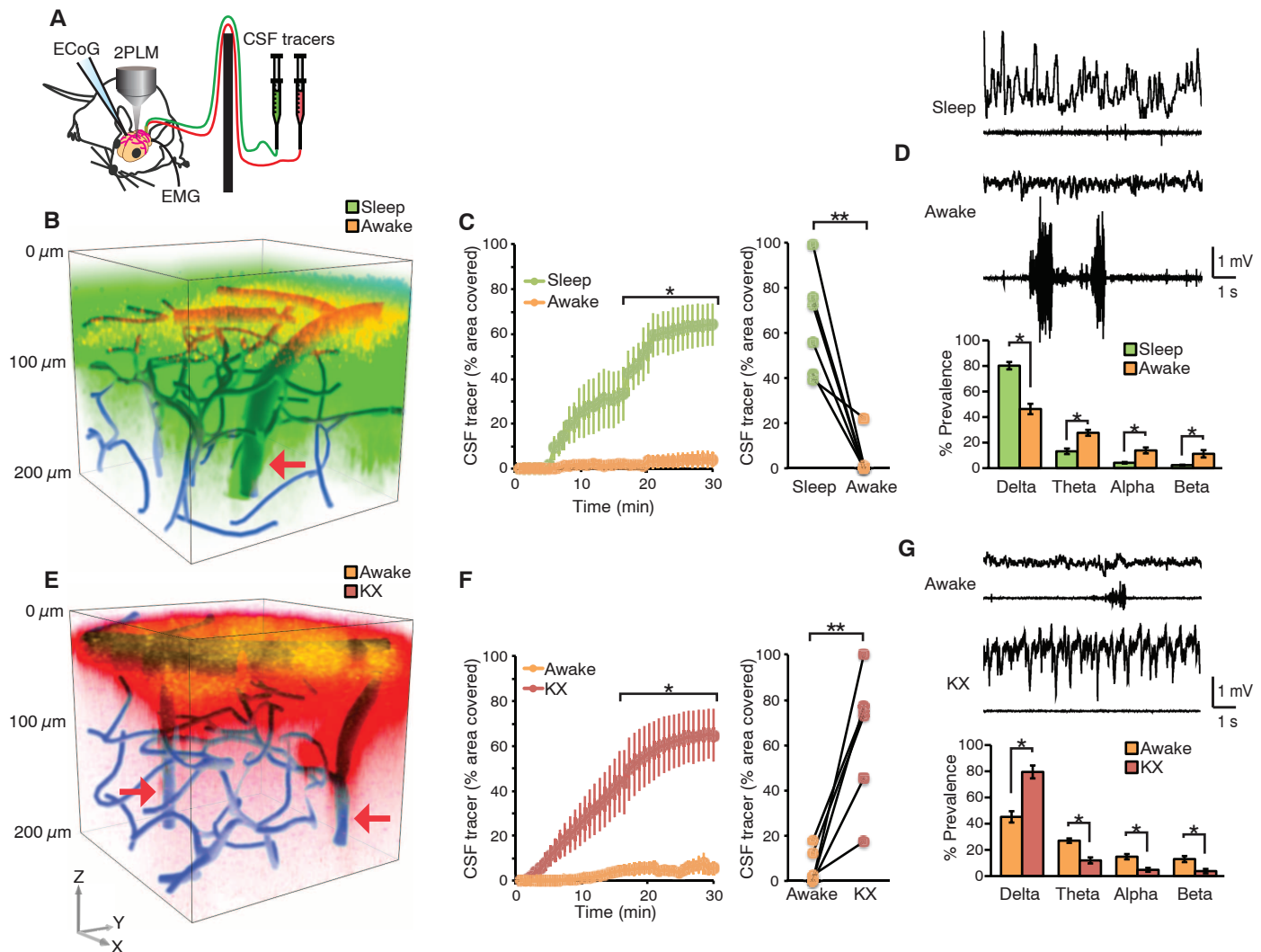


Fig. 1. Wakefulness suppresses influx of CSF tracers. (A) Diagram of experimental setup used for two-photon imaging of CSF tracer movement in real time. To avoid disturbing the state of brain activity, a cannula with dual ports was implanted in the cisterna magna for injection of CSF tracers. ECoG and EMG were recorded to monitor the state of brain activity. (B) Three-dimensional (3D) vectorized reconstruction of the distribution of CSF tracers injected in a sleeping mouse and then again after the mouse was awakened. The vasculature was visualized by means of cascade blue-dextran administered via the femoral vein. FITC-dextran (green) was first injected in the cisterna magna in a sleeping mouse and visualized by collecting repeated stacks of z-steps. Thirty min later, the mouse was awakened by gently moving its tail, and Texas red-dextran (red) was administered 15 min later. The experiments were performed mostly asleep (12 to 2 p.m.). The arrow points to penetrating arteries. (C) Comparison of time-dependent CSF influx in sleep versus awake. Tracer influx was quantified 100 μ m below the cortical surface; $n = 6$ mice; $*P < 0.05$, two-way ANOVA with Bonferroni test. (Right) The tracer

intensity within the two arousal states at the 30-min time point was compared. $**P < 0.01$, t test. (D) ECoG and EMG recordings acquired during sleep and after the mouse was awakened. Power spectrum analysis of all the animals analyzed in the two arousal states ($n = 6$ mice; $*P < 0.05$, t test). (E) 3D reconstruction of CSF tracer influx into the mouse cortex. FITC-dextran was first injected in the awake stage, and cortical influx was visualized by means of two-photon excitation for 30 min. The mouse was then anesthetized with ketamine/xylazine (intraperitoneally), and Texas red-dextran was injected intracranially 15 min later. The vasculature was visualized by means of cascade blue-dextran. Arrows point to penetrating arteries. (F) Comparison of time-dependent CSF influx in awake versus ketamine/xylazine anesthesia; $n = 6$ mice; $*P < 0.05$, two-way ANOVA with Bonferroni test. (Right) The tracer intensity during the two arousal states at the 30-min time point was compared. $**P < 0.01$, t test. (G) ECoG and EMG recordings in the awake mouse and after administration of ketamine/xylazine. Power spectrum analysis of all the animals analyzed in the two arousal states; $n = 6$ mice; $*P < 0.05$, t test.

which eliminated interanimal variability in electrode placement and TMA calibration, showed that anesthesia consistently increased the interstitial space volume fraction by $>60\%$, from $13.6 \pm 1.6\%$ for awake mice to $22.7 \pm 1.3\%$ in the same mice after they received ketamine/xylazine ($n = 10$ mice, $P < 0.01$, paired t test) (Fig. 2D). Analysis of ECoG activity extracted from the TMA reference electrode showed that ketamine/xylazine

increased the power of slow wave activity in all animals analyzed (Fig. 2E). Thus, the cortical interstitial volume fraction is 13 to 15% in the awake state as compared to 22 to 24% in sleeping or anesthetized mice. Tortuosity of the interstitial space did not differ significantly according to changes in the state of brain activity; awake, sleeping, and anesthetized mice all exhibited a λ value in the range of 1.3 to 1.8, which is consistent with

earlier reports ($n = 4$ to 10 mice, $P > 0.1$, t test) (Fig. 2, B and D) (19–21). Recordings obtained 300 μ m below the cortical surface did not differ significantly from those obtained at 150 μ m, suggesting that preparation of the cranial window was not associated with tissue injury ($n = 6$ mice, $P > 0.4$, t test) (Fig. 2D and fig. S3D). Other reports have shown that the interstitial volume is $\sim 19\%$ in anesthetized young mice but declines to $\sim 13\%$ in

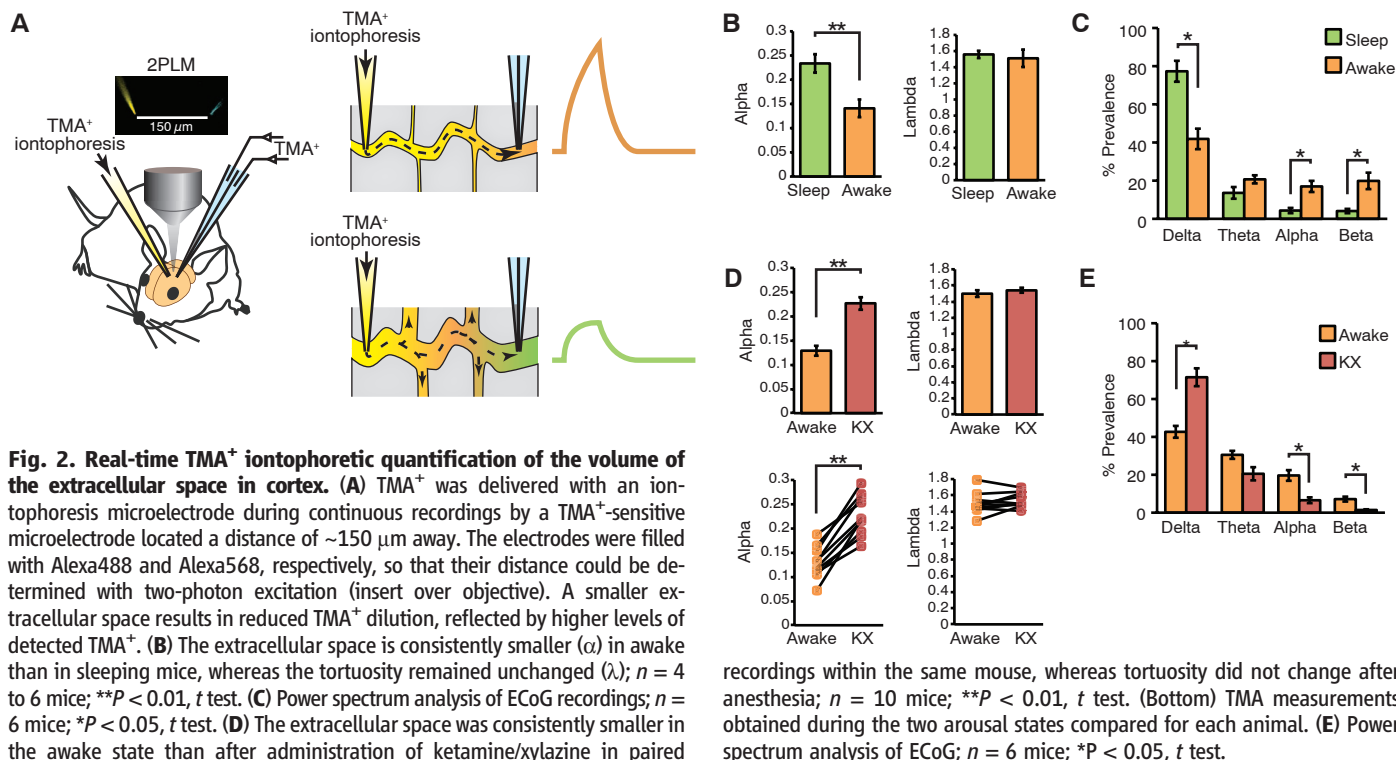
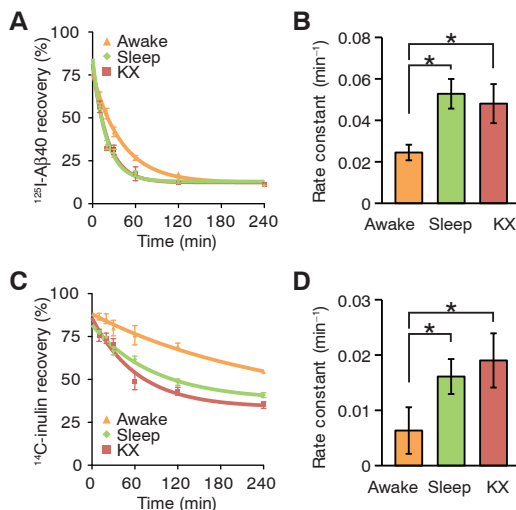


Fig. 3. Sleep improves clearance of A β .

(A) Time-disappearance curves of 125 I-A β_{1-40} after its injection into the frontal cortex in awake (orange triangles), sleeping (green diamonds), and anesthetized (red squares, ketamine/xylozine) mice. (B) Rate constants derived from the clearance curves. (C) Time-disappearance curves of 14 C-inulin after its injection into the frontal cortex of awake (orange triangles), sleeping (green diamonds), and anesthetized (red squares, ketamine/xylozine) mice. (D) Rate constants derived from the clearance curves. A total of 77 mice were included in the analysis: 25 awake, 29 asleep, and 23 anesthetized, with 3 to 6 mice per time point. $*P < 0.05$ compared with awake, ANOVA with Bonferroni test.



aged mice (22). Collectively, these observations support the notion that influx of CSF tracers is suppressed in awake mice as a result of contraction of the interstitial space: The smaller space during wakefulness increases tissue resistance to interstitial fluid flux and inward movement of CSF. This effect of arousal state on interstitial volume likely holds major implications for diffusion of neurotransmitters, such as glutamate (23).

Because previous analysis indicates that as much as 65% of exogenously delivered A β is cleared by the glymphatic system (12), we tested whether interstitial A β is cleared most efficiently during sleep. Radiolabeled 125 I-A β_{1-40} was injected intracortically in three groups of animals: freely

behaving awake mice, naturally sleeping mice, and animals anesthetized with ketamine/xylozine (fig. S4). Brains were harvested 10 to 240 min later for analysis of 125 I-A β retention. A β was cleared twofold faster in the sleeping mice as compared with the awake mice ($n = 23$ to 29 mice, $P < 0.05$, ANOVA with Bonferroni test) (Fig. 3, A and B, $P < 0.05$). A β clearance did not differ between sleeping and anesthetized mice. Because A β is also removed from CNS via receptor-mediated transport across the blood-brain barrier (24), we also analyzed the clearance of an inert tracer, 14 C-inulin. 14 C-inulin was cleared more efficiently (greater than twofold) in sleeping and anesthetized mice as compared with awake mice (Fig. 3, C and D).

What drives the brain state-dependent changes of the interstitial space volume? The observation that anesthesia increases glymphatic influx and efflux (Figs. 1 and 3), suggests that it is not circadian rhythm but rather the sleep-wake state itself that determines the volume of the interstitial space and therefore the efficiency of glymphatic solute clearance. Arousal is driven by the concerted release of neuromodulators (25). In particular, locus coeruleus-derived noradrenergic signaling appears critical for driving cortical networks into the awake state of processing (26, 27). In peripheral tissues, such as kidney and heart, noradrenaline regulates the activity of membrane transporters and channels that control cell volume (28). We hypothesized that adrenergic signaling in the awake state modifies cell volume and thus the size of the interstitial space. We first assessed whether suppression of adrenergic signaling in the awake conscious brain can enhance glymphatic tracer influx by pre-treating awake mice with a cocktail of adrenergic receptor antagonists or vehicle (aCSF) 15 min before infusion of fluorescent CSF tracers (27). The adrenergic receptor antagonists were administered through a cannula inserted into the cisterna magna, with an initial bolus followed by slow continuous drug infusion. Administration of adrenergic antagonists induced an increase in CSF tracer influx, resulting in rates of CSF tracer influx that were more comparable with influx observed during sleep or anesthesia than in the awake state (Fig. 4, A and B, and fig. S5). We asked whether increases in the level of norepinephrine (NE) resulting from stress during restraining at the microscope stage affected the observations. Microdialysis samples

of the interstitial fluid showed that the NE concentration did not increase in trained mice during restraining but that NE, as expected, fell after administration of ketamine/xylazine (Fig. 4C).

We next evaluated whether adrenergic receptor inhibition increased interstitial volume in the same manner as sleep and anesthesia. We used the TMA method to quantify the effect of local adrenergic inhibition on the volume of the interstitial space. To restrict adrenergic inhibition to the cortex, receptor antagonists were applied directly to the exposed cortical surface rather than intracisternal delivery. TMA recordings showed that inhibition of adrenergic signaling in cortex increased the interstitial volume fraction from $14.3 \pm 5.2\%$ to $22.6 \pm 1.2\%$ ($n = 4$ to 8 mice, $P < 0.01$, t test). Interstitial volume was significantly greater than in awake littermates exposed to vehicle (aCSF) ($P < 0.01$) but comparable with the

interstitial volume in sleeping or anesthetized mice ($P = 0.77$ and $P = 0.95$, respectively, t test) (Fig. 4D). Cortical ECoG displayed an increase in the power of slow waves when exposed to adrenergic receptor antagonists ($n = 7$ mice, $P < 0.01$, one-way ANOVA with Bonferroni test). In accordance with earlier findings (27), analysis of the power spectrum showed that inhibition of adrenergic signaling transformed the cortical ECoG of awake mice into a more sleep-like, albeit less regular, profile (Fig. 4E). These analyses suggest that adrenergic signaling plays an important role in modulating not only cortical neuronal activity but also the volume of the interstitial space. NE triggers rapid changes in neural activity (27, 28), which in turn can modulate the volume of the interstitial space volume (29). Nevertheless, additional analysis is clearly required to determine which cell types contribute to expansion of the

interstitial space volume during sleep, anesthesia, or blockade of NE receptors (Figs. 2, B to D, and 4D).

Because of the high sensitivity of neural cells to their environment, it is essential that waste products of neural metabolism are quickly and efficiently removed from the brain interstitial space. Several degradation products of cellular activity, such as A β oligomers and amyloid depositions, have adverse effects on synaptic transmission (30) and cytosolic Ca $^{2+}$ concentrations (31) and can trigger irreversible neuronal injury (32). The existence of a homeostatic drive for sleep—including accumulation of a “need to sleep” substance during wakefulness that dissipates during sleep—has been proposed (33). Because biological activity is inevitably linked to the production of metabolic degradation products, it is possible that sleep subserves the important function of clearing multiple poten-

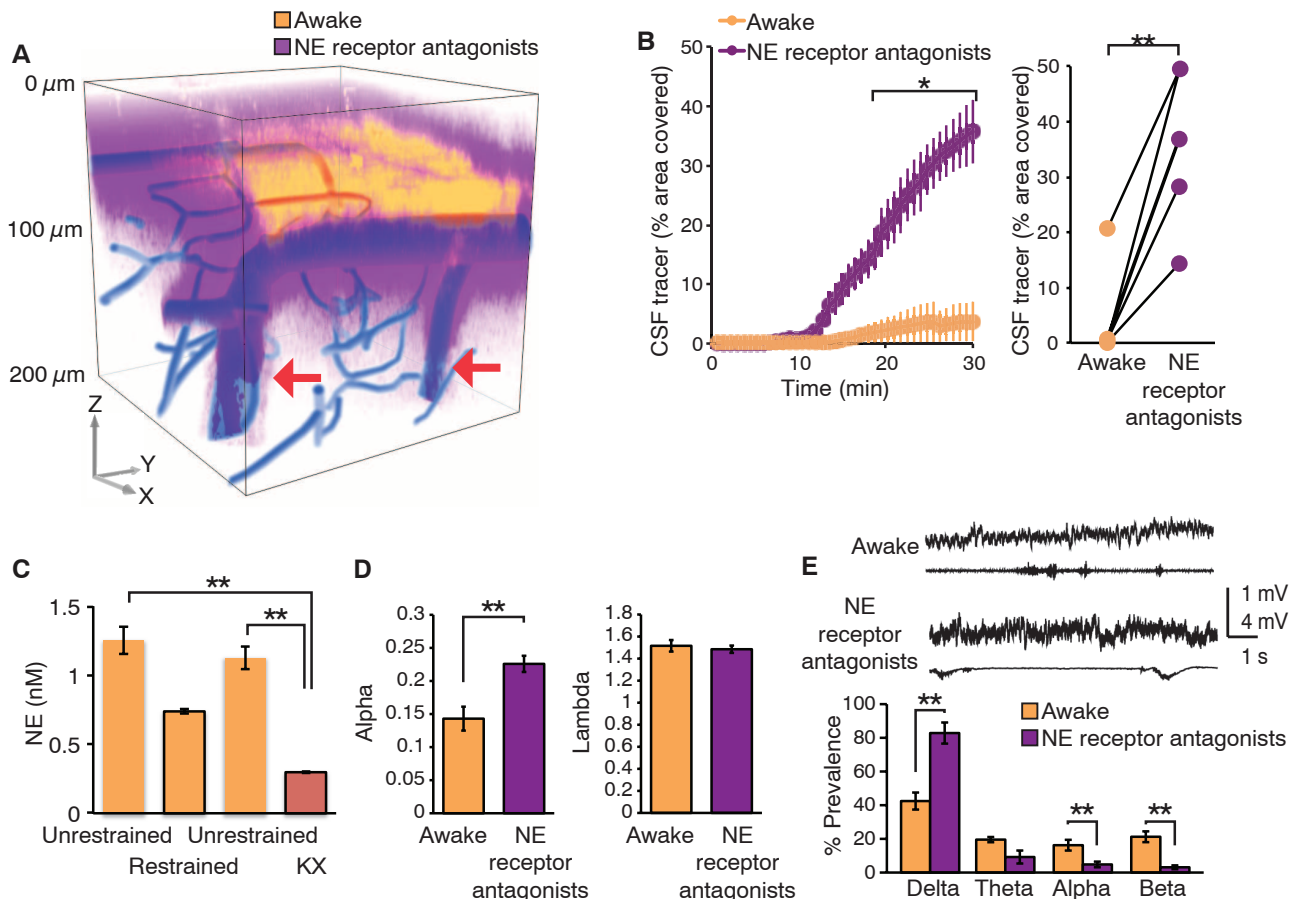


Fig. 4. Adrenergic inhibition increases CSF influx in awake mice. (A) CSF tracer influx before and after intracisternal administration of a cocktail of adrenergic receptor antagonists. FITC-dextran (yellow, 3 kD) was first injected in the cisterna magna in the awake mouse, and cortical tracer influx was visualized by means of two-photon excitation for 30 min. The adrenergic receptor antagonists (prazosin, atipamezole, and propranolol, each 2 mM) were then slowly infused via the cisterna magna cannula for 15 min followed by injection of Texas red-dextran (purple, 3 kD). The 3D reconstruction depicts CSF influx 15 min after the tracers were injected in cisterna magna. The vasculature was visualized by means of cascade blue-dextran. Arrows point to penetrating arteries. (B) Comparison of tracer influx as a function of time before and after administration of adrenergic receptor antagonists. Tracer in-

flux was quantified in the optical section located 100 μm below the cortical surface; $n = 6$ mice; $*P < 0.05$, two-way ANOVA with Bonferroni test. (Right) The tracer intensity during the two arousal states at the 30-min time point was compared. $**P < 0.01$, t test. (C) Comparison of the interstitial concentration of NE in cortex during head-restraining versus unrestrained (before and after), as well as after ketamine/xylazine anesthesia. Microdialysis samples were collected for 1 hour each and analyzed by using high-performance liquid chromatography. $**P < 0.01$, one-way ANOVA with Bonferroni test. (D) TMA $^{+}$ iontophoretic quantification of the volume of the extracellular space before and after adrenergic inhibition; $n = 4$ to 8 mice; $**P < 0.01$, t test. (E) Power spectrum analysis, $n = 7$ mice; $**P < 0.01$, one-way ANOVA with Bonferroni test.

tially toxic CNS waste products. Our analysis indicates that the cortical interstitial space increases by more than 60% during sleep, resulting in efficient convective clearance of A β and other compounds (Figs. 2 and 3). The purpose of sleep has been the subject of numerous theories since the time of the ancient Greek philosophers (34). An extension of the findings reported here is that the restorative function of sleep may be due to the switching of the brain into a functional state that facilitates the clearance of degradation products of neural activity that accumulate during wakefulness.

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Supplementary Materials

www.sciencemag.org/content/342/6156/373/suppl/DC1
Materials and Methods
Figs. S1 to S5
References

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Reading Literary Fiction Improves Theory of Mind

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Understanding others' mental states is a crucial skill that enables the complex social relationships that characterize human societies. Yet little research has investigated what fosters this skill, which is known as Theory of Mind (ToM), in adults. We present five experiments showing that reading literary fiction led to better performance on tests of affective ToM (experiments 1 to 5) and cognitive ToM (experiments 4 and 5) compared with reading nonfiction (experiments 1), popular fiction (experiments 2 to 5), or nothing at all (experiments 2 and 5). Specifically, these results show that reading literary fiction temporarily enhances ToM. More broadly, they suggest that ToM may be influenced by engagement with works of art.

The capacity to identify and understand others' subjective states is one of the most stunning products of human evolution. It allows successful navigation of complex social relationships and helps to support the empathic responses that maintain them (1–5). Deficits in this set of abilities, commonly referred to as Theory of Mind (ToM), are associated with psychopathologies marked by interpersonal difficulties (6–8). Even when the ability is intact, disengagement of ToM has been linked to the breakdown of positive interpersonal and intergroup relationships (9).

Researchers have distinguished between affective ToM (the ability to detect and understand others' emotions) and cognitive ToM (the inference and representation of others' beliefs and in-

tentions) (7, 8). The affective component of ToM, in particular, is linked to empathy (positively) and antisocial behavior (negatively) (7, 8). It is thus not surprising that we foster ToM in our children by having them attend to the emotional states of others: “Do you think he is happy or sad as a consequence of your action?” Such explicit encouragements to understand others usually diminish when children appear to skillfully and empathically engage in interpersonal relationships. Cultural practices, though, may function to promote and refine interpersonal sensitivity throughout our lives. One such practice is reading fiction.

Familiarity with fiction, self-reported empathy, and performance on an advanced affective ToM test have been correlated (10, 11), and limited experimental evidence suggests that reading fiction increases self-reported empathy (12, 13). Fiction seems also to expand our knowledge of others' lives, helping us recognize our similarity to them (10, 11, 14). Although fiction

may explicitly convey social values and reduce the strangeness of others, the observed relation between familiarity with fiction and ToM may be due to more subtle characteristics of the text. That is, fiction may change how, not just what, people think about others (10, 11, 14). We submit that fiction affects ToM processes because it forces us to engage in mind-reading and character construction. Not any kind of fiction achieves that, though. Our proposal is that it is literary fiction that forces the reader to engage in ToM processes.

The category of literary fiction has been contested on the grounds that it is merely a marker of social class, but features of the modern literary novel set it apart from most best-selling thrillers or romances. Miall and Kuiken (15–17) emphasize that through the systematic use of phonological, grammatical, and semantic stylistic devices, literary fiction defamiliarizes its readers. The capacity of literary fiction to unsettle readers' expectations and challenge their thinking is also reflected in Roland Barthes's (18) distinction between writerly and readerly texts. Although readerly texts—such as most popular genre fiction—are intended to entertain their mostly passive readers, writerly—or literary—texts engage their readers creatively as writers. Similarly, Mikhail Bakhtin (19) defined literary fiction as polyphonic and proposed that readers of literary fiction must contribute their own to a cacophony of voices. The absence of a single authorial perspective prompts readers to enter a vibrant discourse with the author and her characters.

Bruner (20), like Barthes and Bakhtin, has proposed that literature engages readers in a discourse that forces them to fill in gaps and search “for meanings among a spectrum of possible meanings” (p. 25). Bruner argues that to elicit

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this writerly stance, literary fiction triggers presupposition (a focus on implicit meanings), subjectification [depicting reality “through the filter of the consciousness of protagonists in the story” (p. 25)], and multiple perspectives (perceiving the world simultaneously from different viewpoints). These features mimic those of ToM.

Our contention is that literary fiction, which we consider to be both writerly and polyphonic, uniquely engages the psychological processes needed to gain access to characters’ subjective experiences. Just as in real life, the worlds of literary fiction are replete with complicated individuals whose inner lives are rarely easily discerned but warrant exploration. The worlds of fiction, though, pose fewer risks than the real world, and they present opportunities to consider the experiences of others without facing the potentially threatening consequences of that engagement. More critically, whereas many of our mundane social experiences may be scripted by convention and informed by stereotypes, those presented in literary fiction often disrupt our expectations. Readers of literary fiction must draw on more flexible interpretive resources to infer the feelings and thoughts of characters. That is, they must engage ToM processes. Contrary to literary fiction, popular fiction, which is more readerly, tends to portray the world and characters as internally consistent and predictable (21). Therefore, it may reaffirm readers’ expectations and so not promote ToM.

To test our general hypothesis that literary fiction would prime ToM, we first compared the effects of reading literary fiction with reading nonfiction (experiment 1) and then focused on testing our predictions about the different effects of reading literary and popular fiction (experiments 2 to 5).

Difficulty in precisely quantifying literariness notwithstanding, some works are considered particularly good examples of literature and are recognized with prestigious awards (e.g., the National Book Award). Although selected through an inherently inexact process, prize-winning texts are more likely to embody general characteristics of literature than bestsellers of genre fiction (e.g., romance and adventure stories). In the absence of a clear means of quantifying literariness, the judgments of expert raters (i.e., literary prize jurors) were used. Accordingly, to study the effects of reading literary fiction, we selected literary works of fiction by award-winning or canonical writers and compared their effects on ToM with reading nonfiction, popular fiction, or nothing at all.

In experiment 1 (22), 86 participants were randomly assigned to read one of six short texts (three literary fiction and three nonfiction). Next, participants completed a false-belief test as a measure of cognitive ToM (23) and an advanced affective ToM test, the Reading the Mind in the Eyes Test [RMET (6)], in which they were asked to identify facially expressed emotions. Participants’ familiarity with fiction was assessed using the Author Recognition Test (24), an index of general exposure

to fiction that avoids problems of socially desirable responding. Affect (25), engagement with the text (transportation scale) (26), and demographic information were assessed.

For the cognitive ToM task, participants were asked to indicate the probability that a character would act according to the character’s own false belief or the participant’s true belief. Participants ($n = 13$) who failed to give probabilities and univariate outliers (>3.5 SD from the mean; $n = 6$) were excluded from the analysis. Probabilities were compared in a 2(false-belief versus no false-belief condition) $\times$ 2(fiction versus nonfiction) analysis of variance (ANOVA). There was no main effect for the type of scenario, which suggests no evidence of egocentric bias ($F_{1,63} = 1.47$, $P = 0.22$). The level of false estimates was low across conditions (grand mean $\pm$ standard deviation, 6.61 ± 9.79).

Scores for the affective ToM task were computed by summing the number of correct identifications of facially expressed emotions (6) and analyzed using ANOVA, with condition and Author Recognition Test as between-participants factors (Table 1). Scores were higher in the literary fiction than nonfiction condition (Table 2). Higher Author Recognition Test scores (indicating more familiarity with fiction) predicted higher RMET scores. When entered as covariates, education; gender; age; transportation; negative affect; self-reported sadness; and average time spent on RMET items did not significantly alter the main effect of condition ($P = 0.05$). More time spent on RMET items predicted better performance ($\beta = 0.23$, $P = 0.02$). No other covariates approached significance (P values of >0.14).

Experiment 2 aimed to replicate and extend the findings of experiment 1 by using different texts and a different measure of affective ToM, the Diagnostic Analysis of Nonverbal Accuracy 2—Adult Faces test (DANVA2-AF) (27). Experiment 2 was also designed to directly differentiate between the effects of popular versus literary fiction (28).

Participants ($n = 114$) were randomly assigned to read one of three excerpts from recent

finalists for the National Book Award (literary fiction condition), one of three excerpts from recent bestsellers on Amazon.com (popular fiction condition), or nothing at all (no-reading condition) (22). Participants then completed the measure of cognitive ToM used in experiment 1 and the DANVA2-AF before completing the Author Recognition Test, the transportation scale, and demographic questions. Performance on the false-belief cognitive ToM task was analyzed as in experiment 1, but no significant effects were detected (P values of >0.13).

DANVA2-AF scores were computed by summing errors on all of the negative affect items (22). Untransformed means are reported, but log-transformed scores were used in an ANOVA with experimental condition and Author Recognition Test as between-participants factors (see Table 1). No interaction emerged, but higher scores on the Author Recognition Test were weakly associated with fewer errors on the DANVA2-AF. The omnibus main effect of condition was marginally significant, and the pairwise comparisons revealed significant differences between conditions consistent with our hypothesis. Fewer errors were made in the literary fiction condition than in the no-reading and popular fiction conditions, whereas there was no difference between the latter two ($P = 0.98$) (see Table 2). As in experiment 1, education; gender; and age were not significant covariates (P values of >0.34) and did not alter the critical, omnibus main effect of condition ($P = 0.08$). Transportation did not correlate with DANVA2-AF scores ($P = 0.94$).

Experiment 3 ($N = 69$) aimed to replicate the literary fiction versus popular fiction comparison (22). The popular fiction texts were three stories from an edited anthology of popular fiction (29), and literary fiction texts were three stories from a collection of the 2012 PEN/O. Henry Award winners for short stories (30). Participants’ affect was assessed using the Positive Affect Negative Affect Scale (PANAS) and a single-item report of sadness. Using the same analytical strategy used in experiment 1, it was found that RMET scores

Table 1. RMET and DANVA2-AF analyses.

Experiment	Independent variable	Test	<i>P</i>	ω_p^2
Exp. 1 RMET	Condition	$F_{1,82} = 6.40$	0.01	0.05
	Author Recognition Test	$\beta = 0.36$	0.0003	0.13
	Author Recognition Test $\times$ Condition	$F_{1,82} = 1.06$	0.30	0.00
Exp. 2 DANVA2-AF	Condition	$F_{2,108} = 2.57$	0.08	0.02
	Author Recognition Test	$\beta = -0.16$	0.08	0.01
	Author Recognition Test $\times$ Condition	$F_{2,108} = 1.17$	0.31	0.00
Exp. 3 RMET	Condition	$F_{1,65} = 4.07$	0.04	0.04
	Author Recognition Test	$\beta = -0.01$	0.90	-0.01
	Author Recognition Test $\times$ Condition	$F_{1,65} = 0.01$	0.90	-0.01
Exp. 4 RMET	Condition	$F_{1,68} = 4.39$	0.04	0.04
	Author Recognition Test	$\beta = 0.39$	<0.001	0.15
	Author Recognition Test $\times$ Condition	$F_{1,68} = 1.50$	0.22	0.00
Exp. 5 RMET	Condition	$F_{2,352} = 3.10$	0.04	0.01
	Author Recognition Test	$\beta = 0.28$	<0.001	0.07
	Author Recognition Test $\times$ Condition	$F_{2,352} = 1.37$	0.25	0.00

were higher in the literary fiction condition than in the popular fiction condition. There were no effects involving the Author Recognition Test (for test, Table 1; for means, Table 2). Education, gender, and the average time spent on RMET items were not significant covariates (P values of >0.12) and did not alter the effect of condition ($P = 0.04$).

In experiments 1 and 2, no effects were observed on the cognitive ToM measure, a false-belief task. Since participants in neither condition clearly failed to recruit cognitive ToM, it is possible that the task may have been insufficiently sensitive. Therefore a fourth experiment included the Yoni test (7). The Yoni test is a new measure that has been used in only a handful of studies. However, it has been validated (7, 8, 31) and has the advantage of assessing both cognitive and affective ToM.

In experiment 4, four of the texts used in experiment 3 along with two new stories, one for each condition (i.e., literary fiction and popular fiction), from the same sources were used (22). Participants ($N = 72$) completed the RMET and the Yoni test. For the 24 cognitive and 24 affective ToM trials in the Yoni test, participants must draw from minimal linguistic and visual cues to infer a character's thoughts and emotions, respectively. An additional 16 control trials require the identification of spatial relations. For each type of item, there are equal numbers of trials requiring first-order and second-order (more difficult) inferences.

RMET scores were higher in the literary fiction condition than in the popular fiction condition (for tests, see Table 1; for means, see Table 2). Author Recognition Test scores predicted RMET scores. Entered as covariates, subject variables (i.e., education, age, and gender) did not reach significance (P values of >0.14), though time spent on RMET items did ($\beta = 0.21$, $P = 0.04$). However, the effect of condition was only slightly altered and remained significant ($P = 0.05$).

Yoni performance was analyzed via a mixed analysis of covariance (ANCOVA) with type (affective versus cognitive) and level of difficulty (first order versus second order) of trials as within-participants factors, condition and Author Recognition Test scores as between-participants factors,

and scores on the control task as a covariate (31). A main effect of condition emerged ($F_{1,67} = 4.47$, $P = 0.03$, $\omega_p^2 = 0.04$) but no other effects involving condition or Author Recognition Test scores approached significance (P values of >0.27). Other significant effects, which are not relevant to the hypotheses, are described in the supplementary materials (22). Participants in the literary fiction condition [0.89 ± 0.08 , 95% confidence interval (CI) = 0.86, 0.92] performed with greater accuracy on all ToM trials than those in the popular fiction condition (0.85 ± 0.10 , CI = 0.82, 0.87).

A fifth experiment (22) aimed to replicate experiment 4 and test for the influences of subject variables (i.e., education, age, gender) and possible confounds with a larger sample ($N = 356$). As in experiments 3 and 4, three works of literary fiction were taken from a collection of the 2012 PEN/O. Henry Prize winners (30) and three works of popular fiction from an anthology (29). Participants were randomly assigned to the literary fiction, popular fiction, or no-reading control condition; completed the RMET and Yoni tasks; reported their current affect (PANAS), along with two additional items assessing sadness and happiness; and completed the Author Recognition Test. Participants in the two reading conditions completed the transportation scale and two additional items assessing the extent to which they enjoyed reading the text and how much they thought it represented "excellent literature." All participants reported their age, gender, ethnicity, and highest level of attained education before being debriefed and compensated.

Literary texts (3.54 ± 1.31 , CI = 3.28, 3.80) were enjoyed less than popular texts (4.07 ± 1.53 , CI = 3.80, 4.34; $F_{1,223} = 7.62$, $P = 0.006$, $\omega_p^2 = 0.02$), but they were seen as better examples of literature (4.84 ± 1.40 , CI = 4.56, 5.11) than popular texts (4.43 ± 1.60 , CI = 4.15, 4.72; $F_{1,223} = 4.04$, $P = 0.04$, $\omega_p^2 = 0.01$). Reported transportation did not significantly differ across conditions ($F_{1,223} = 3.20$, $P = 0.07$, $\omega_p^2 = 0.00$), although it was slightly higher in the literary condition (3.90 ± 0.41 , CI = 3.83, 3.98) than the popular condition (3.81 ± 0.39 , CI = 3.73, 3.88). None of these variables were correlated (P values of

>0.11) with performance on either the RMET or the Yoni task (controlling for performance on physical trials).

Results on the RMET were analyzed as in the previous experiments. The effect of condition was significant (see Table 1). Scores were significantly higher in the literary fiction condition than in the popular fiction and no-reading conditions (see Table 2). The latter two conditions did not differ ($P = 0.65$). A significant main effect of Author Recognition Test scores emerged (see Table 1). Added as covariates, gender, education, age, positive affect, negative affect, sadness, happiness, and time spent on RMET items did not significantly relate to RMET scores (P values of >0.23), and the effect of condition was only slightly altered ($P = 0.06$).

The analytical strategy used in experiment 4 was also used for the Yoni task. The main effect of condition ($F_{2,351} = 0.64$, $P = 0.52$) was not significant, but there was a significant interaction of condition and the two within-subjects factors, trial difficulty and trial type ($F_{2,351} = 3.42$, $P = 0.03$). The interaction of Author Recognition Test scores, trial difficulty, and trial type approached significance ($F_{1,351} = 2.88$, $P = 0.09$), but no other effects involving condition or Author Recognition Test did (P values of >0.11). Other significant effects, which are not relevant to the hypotheses, are described in the supplementary materials (22). To disentangle the three-way interaction including the experimental condition, a repeated measures ANCOVA, with item type (cognitive, affective) and condition as factors, and performance on the control task as covariate, was conducted separately for first-order and second-order trials. On first-order trials, there was a main effect of the covariate ($\beta = 0.20$, $P < 0.001$, $\omega_p^2 = 0.03$) and of condition ($F_{2,351} = 4.21$, $P = 0.01$, $\omega_p^2 = 0.01$). No other effects approached significance (P values of >0.87). Pair-wise comparisons revealed that scores were higher in the literary fiction condition (0.98 ± 0.02 , CI = 0.97, 0.99) than in the popular fiction condition (0.96 ± 0.06 , CI = 0.95, 0.97; $t = 2.85$, $P = 0.004$) and the no-reading condition (0.97 ± 0.05 , CI = 0.96, 0.98; $t = 2.01$, $P = 0.04$). The popular fiction condition and no-reading condition did not differ ($P = 0.33$). On second-order trials, no effects involving condition or Author Recognition Test scores approached significance (P values of >0.16).

The difference between first- and second-order trials, which appeared only in experiment 5, might be due to its higher statistical power, which allowed for this difference to be detected. The second-order Yoni trials may require a set of more advanced cognitive skills (e.g., metarepresentation) that are less easily influenced by manipulation than the other tasks, all of which are first-order ToM tasks.

Experiment 1 showed that reading literary fiction, relative to nonfiction, improves performance on an affective ToM task. Experiments 2 to 5 showed that this effect is specific to literary fiction. On

Table 2. Means (adjusted for other terms in the models) and standard deviations of RMET and DANVA2-AF scores. 95% confidence intervals are reported in brackets. X, no data. Means in the same row that share the same superscripts differ at $P < 0.05$.

Experiment	Literary fiction	Popular fiction	No reading	Nonfiction
Exp. 1 RMET	25.90 $\pm$ 4.38 ^a [24.55, 27.24]	X	X	23.47 $\pm$ 5.17 ^a [22.13, 24.82]
Exp. 2 DANVA2-AF	4.70 $\pm$ 2.31 ^{ab} [3.79, 5.61]	5.85 $\pm$ 2.93 ^a [4.96, 6.74]	5.86 $\pm$ 2.89 ^b [5.00, 6.72]	X
Exp. 3 RMET	25.92 $\pm$ 4.07 ^a [23.99, 27.86]	23.22 $\pm$ 6.16 ^a [21.34, 25.09]	X	X
Exp. 4 RMET	26.19 $\pm$ 5.43 ^a [24.52, 27.85]	23.71 $\pm$ 5.08 ^a [22.18, 25.24]	X	X
Exp. 5 RMET	26.21 $\pm$ 3.59 ^{ab} [25.45, 26.97]	24.96 $\pm$ 4.60 ^a [24.18, 25.74]	25.20 $\pm$ 4.69 ^b [24.99, 25.91]	X

cognitive measures, no effects emerged on the false-belief task used in experiments 1 and 2. Because error rates on the false-belief task were very low, the measure may have been insufficiently sensitive to capture the effects of the manipulations. However, on the more-demanding Yoni task used in experiments 4 and 5, the effect on cognitive trials was present and indistinguishable from that on affective trials.

The Author Recognition Test predicted RMET scores in experiments 1, 4, and 5, and success on the DANVA2-AF (marginally) in experiment 2; but it did not predict performance on the Yoni task or, anomalously, the RMET in experiment 3. Thus, although generally consistent with previous findings (10–12), our pattern of results suggests the need for further research into the relation between measures of familiarity with fiction and performance on different ToM tasks.

The results of five experiments support our hypothesis that reading literary fiction enhances ToM. Existing explanations focused on the content of fiction cannot account for these results. First, the texts we used varied widely in subject matter. Second, it is unlikely that people learned much more about others by reading any of the short texts. Third, the effects were specific to literary fiction. We propose that by prompting readers to take an active writerly role to form representations of characters' subjective states, literary fiction recruits ToM. The evidence we report here is consistent with this view, but we see these findings as preliminary and much research is needed.

First, our findings demonstrate the short-term effects of reading literary fiction. However, taken together, the relation between the Author Recognition Test and ToM performance and the finding that it is specifically literary fiction that facilitates ToM processes suggest that reading literary fiction may lead to stable improvements in ToM. Because the Author Recognition Test does not distinguish between exposure to literary and popular fiction, additional research with refined methods is necessary to test this hypothesis.

Second, literary fiction, like many stimuli drawn from the real world, is heterogeneous and complex. Although it is not clearly quantifiable, literariness has ecological validity as a construct, as suggested by participants' agreement with prize jurors on the literariness of the texts in experiment 5. On the basis of strategies used by researchers studying violent video games [e.g., (32)] and fiction (12), literariness was held relatively constant in each condition while potentially confounding features varied. Self-reported affect along with transportation into, enjoyment, and perceived literariness of the texts did not account for the effects of condition. Further analyses tested the roles of superficial linguistic features of the texts. Frequencies of negative and positive emotion terms, social words, cognitive words, big words (more than six letters), and self-references were computed in each text using Linguistic Inquiry and Word Count (LIWC) software (33). Stan-

dardized RMET or DANVA2-AF scores from all experiments were analyzed using ANCOVA, with experimental condition and Author Recognition Test scores as factors and all six LIWC variables as covariates (data from the no-reading conditions was not included). The frequency of negative emotion words ($\beta = 0.09$, $P = 0.05$, $\omega_p^2 = 0.00$) positively predicted ToM scores, but no other effects of LIWC variables approached significance (P values of >0.17). The main effects of condition ($F_{1,515} = 12.02$, $P < 0.001$, $\omega_p^2 = 0.02$) and Author Recognition Test scores ($\beta = 0.23$, $P < 0.001$, $\omega_p^2 = 0.05$) remained significant. This result suggests that the effect of literature observed across experiments may not be easily reduced to superficial linguistic characteristics. Future research, notably following the lead of Miall and Kuiken (15–17), as well as Bruner (20), may reveal more subtle, but nonetheless quantifiable, features that set literary fiction apart.

The present findings mark only one step toward understanding the impact of our interactions with fiction, the experiences of which are thought to contribute to the development of consciousness and to enrich our daily lives (34). Indeed, there are surely many consequences of reading on cognitive and affective processes that are independent of its effects on ToM, and it seems likely that many of those may result from popular, as well as literary, fiction. Similarly, whereas literary fiction appears able to promote ToM, this capacity does not fully capture the concept of literariness, which includes, among others, aesthetic and stylistic matters not addressed in this research. It is our hope that further research will focus on other forms of art, such as plays and movies, that involve identifying and interpreting the subjective experiences of others (10, 28).

Literature has been deployed in programs intended to promote social welfare, such as those intended to promote empathy among doctors (35) and life skills among prisoners (36). Literature is, of course, also a required subject throughout secondary education in the United States, but reformers have questioned its importance: A new set of education standards that has been adopted by 46 U.S. states (the Common Core State Standards) controversially calls for less emphasis on fiction in secondary education [see (37)]. Debates over the social value of types of fiction and the arts more broadly are important, and it seems critical to supplement them with empirical research. These results show that reading literary fiction may hone adults' ToM, a complex and critical social capacity.

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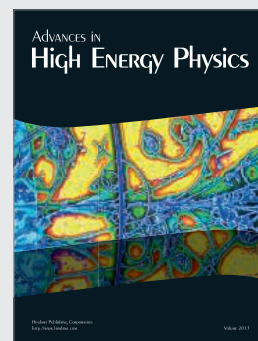
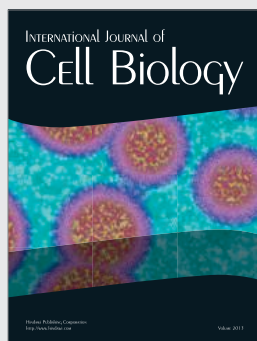
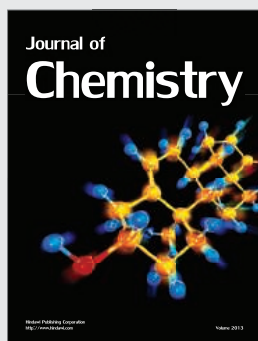
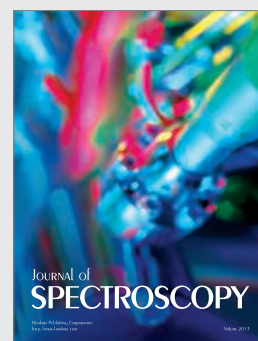
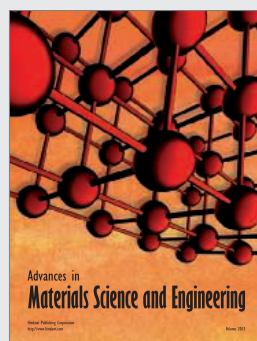
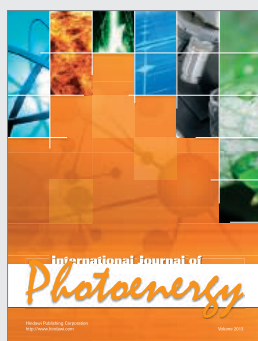
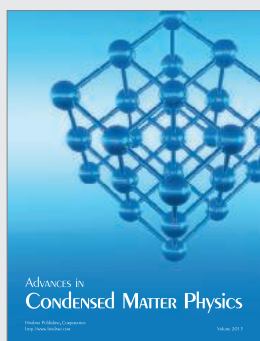
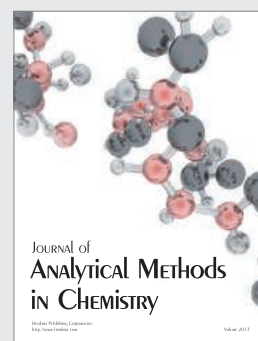
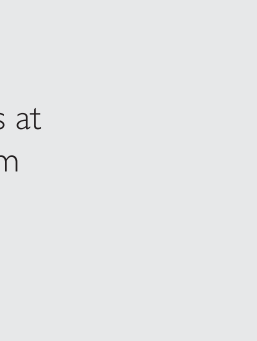
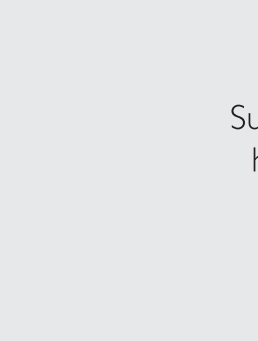
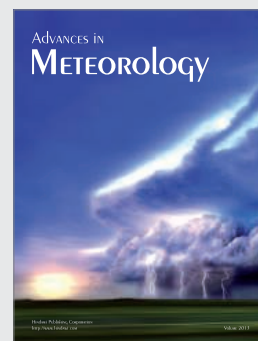
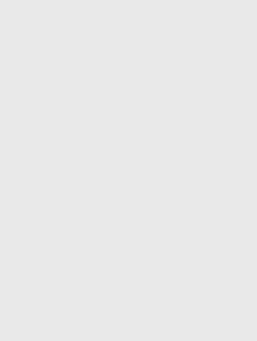
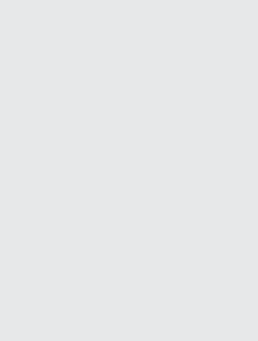
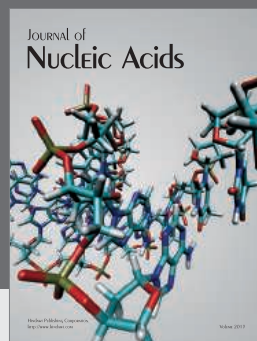
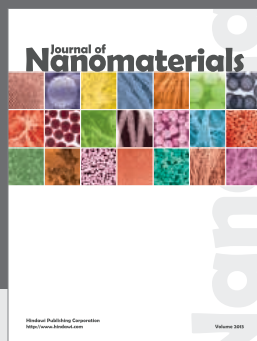
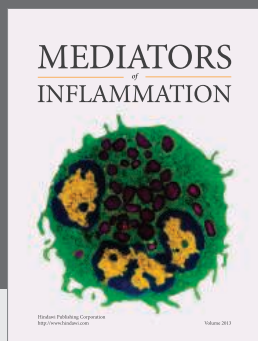
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Supplementary Materials

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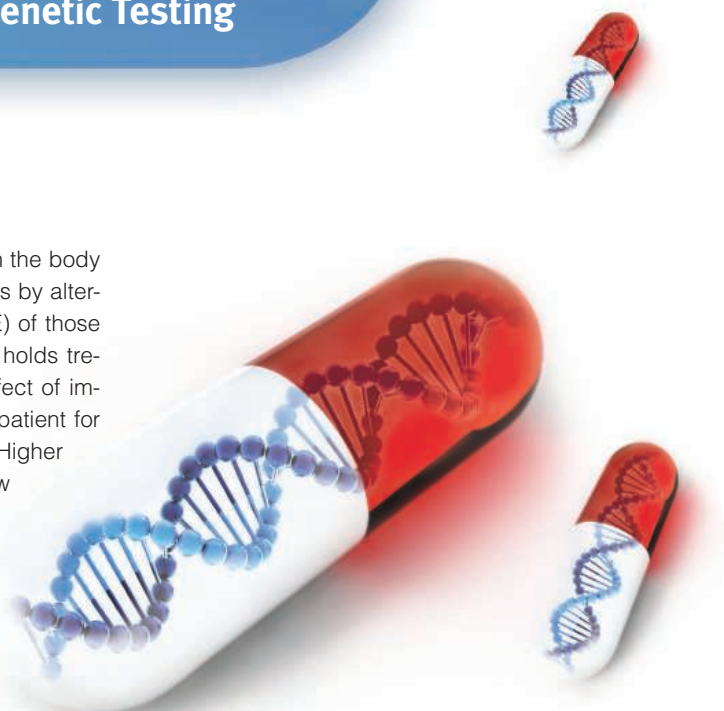
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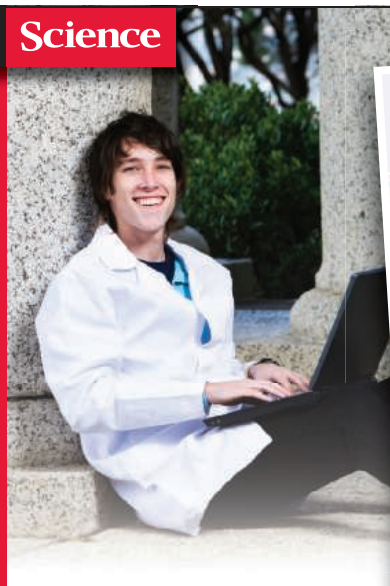
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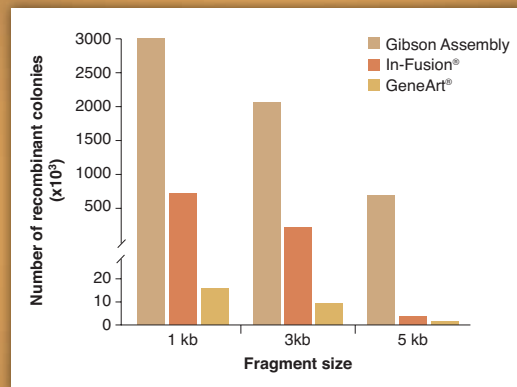
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in Structural Biology**

The Department of Biological Sciences and the Markey Center for Structural Biology at Purdue University invite applications for a tenure-track faculty position in Structural Biology. We expect to fill this academic-year appointment at the Assistant Professor level, but appointment at the Associate level will also be considered. Applicants must have a Ph.D. or equivalent in biology, chemistry, physics or related field and at least 2 years of postdoctoral experience in X-ray crystallography, cryo-EM, NMR or other relevant field. The successful applicant will be required to establish a strong and independent research program in structural biology and to teach at the undergraduate and graduate levels. Applicants with research interests in cancer biology, drug discovery, energy & signal transduction, infectious disease, large macromolecular complexes, membrane-based transport, molecular pathogenesis, neurobiology, viruses or viral-host interactions are encouraged to apply.

The Markey Center for Structural Biology at Purdue is recognized worldwide for its leadership in structural biology of viruses, membrane proteins, receptors, signaling proteins and enzymes in addition to methods development in crystallography, NMR and electron microscopy. The Department has over 50 faculty members conducting research in a wide range of fields including those areas listed above, bioinformatics, computational chemistry and biology, ecology, evolutionary biology and vision research. The successful candidate will have opportunities to interact with colleagues in many departments and colleges including Chemistry, Medicinal Chemistry and Molecular Pharmacology, Computer Science and Physics, and to join centers such as the Center for Cancer Research and the new Center for Drug Discovery. The successful candidate will also have laboratory space in the newly constructed Hockmeyer Hall of Structural Biology and will have access to state-of-the-art shared resources across Purdue including a Titan Krios cryo-TEM, Bruker Avance-III 800 MHz NMR, Rigaku X-ray generators and detectors, and other advanced biophysical instrumentation available at the Bindley Bioscience Center and the Birk Nanotechnology Center.

Applications must be submitted electronically to <https://hiring.science.purdue.edu> as a PDF file that includes a detailed curriculum vitae, names and addresses of three referees, a 2 - 3 page summary of research interests, and a one-page statement on a vision for teaching. Inquiries should be directed to **Structural Biology Search Committee, Department of Biological Sciences, Purdue University, 915 W. State St., West Lafayette, IN 47907-2054**. Review of applications will begin **November 1, 2013** and continue until the position is filled. A background check will be required for employment in this position. Further information about the Department is available at <http://www.bio.purdue.edu/>.

Purdue University is an Equal Opportunity/Equal Access/Affirmative Action Employer fully committed to achieving a diverse workforce.



COLUMBIA | ENGINEERING

The Fu Foundation School of Engineering and Applied Science

Open-rank Faculty Positions in All Departments

Columbia Engineering—The Fu Foundation School of Engineering and Applied Science at Columbia University in New York City invites applications for tenured or tenure-track faculty positions. Positions are available in all nine academic departments:

- Applied Physics and Applied Mathematics
- Biomedical Engineering
- Chemical Engineering
- Civil Engineering and Engineering Mechanics
- Computer Science
- Earth and Environmental Engineering
- Electrical Engineering
- Industrial Engineering and Operations Research
- Mechanical Engineering

Applicants can consult www.engineering.columbia.edu for more information about the School, its departments, and major interdisciplinary institutes, including the Institute for Data Sciences and Engineering and the Zuckerman Mind, Brain, Behavior Institute. Applications exploring the synergy between the departments and these multidisciplinary initiatives are welcome.

Successful candidates should contribute to the advancement of the School by developing a very successful, externally funded research program, being a thought leader in the profession, enhancing the undergraduate and graduate educational mission of the School, and providing active service to professional societies. The candidate is expected to establish multidisciplinary research and educational collaborations across academic departments and units at Columbia University. The School is especially interested in qualified candidates who can contribute, through their research, teaching, and/or service, to the diversity and excellence of the academic community.

Candidates must have a Ph.D. or its professional equivalent by the starting date of the appointment. Applicants for this position at the Assistant Professor level and Associate Professors without tenure must have the potential to do pioneering research and to teach effectively. Applicants for this position at the tenured level (Associate or Full Professor) must have a demonstrated record of outstanding research accomplishments, excellent teaching credentials, and established leadership in the field.

For a listing of ongoing recruiting opportunities, please see <http://engineering.columbia.edu/faculty-job-opportunities>.

*Columbia University is an affirmative action/equal opportunity employer,
with a strong commitment to the quality of faculty life.*



The International Post-Doc Initiative (IPODI) of the TU Berlin aims at increasing the number of female researchers in leadership positions and awards

Seven Incoming Postdoctoral Fellowships
(salary scale between E 13 and E 14 TV-L)

IPODI is open to outstanding researchers of all nationalities and from all fields of research represented at the TU Berlin.

IPODI Fellows will receive:

- A two-year employment contract including social security, health insurance and pension coverage,
- A mobility allowance, a travel allowance and a monthly contribution to research costs,
- The opportunity for professional training in career development and research management.

Conditions for application:

- Applicants must have at least two and not more than ten years of research experience after obtaining their doctoral degree, in exceptional cases an extension of up to three years is possible,
- Applicants must not have resided in Germany for more than 12 months in the three years prior to the call deadline,
- The research proposal has to be supported by a cooperating professor of the TU Berlin.

Applications have to be submitted online via the IPODI Application Portal. For full call for applications and further information see www.ipodi.tu-berlin.de or contact the IPODI Office (application@ipodi.de).

Application deadline: January 15, 2014.

IPODI is co-funded by the Marie Curie Programme of the European Union.



Children's
of Alabama

PEDIATRIC Infectious Diseases

Open Rank, Open Tenure. The Division of Infectious Diseases in the Department of Pediatrics is seeking applications from highly qualified M.D., M.D./Ph.D. or Ph.D., for faculty positions. The Division of Infectious Diseases has a long history of NIH supported laboratory and clinical research. The selected individuals will work collaboratively to expand current research programs as well as establish new areas of research in respiratory viruses and bacterial infections. These positions will be provided generous start-up packages as well as secondary appointment in basic science department and Centers of Excellence at UAB to further the capacity of research in infectious diseases at UAB.

QUALIFICATIONS

The successful candidates for these positions must have a successful record in research with current NIH funding. In addition, the ideal candidates should possess:

- Experience in the academic medical center setting
- A proven track record of research initiation
- Demonstrated research collaboration skills
- Strong communication skills and the highest of ethical standards

Interested applicants should contact:

William Britt, M.D.

Professor, Division of Ped Infectious Diseases

1600 7th Avenue South, CHB #107

Birmingham, AL 35233

Email: wbritt@peds.uab.edu

UAB is an Equal Opportunity/Affirmative Action Employer committed to fostering a diverse, equitable and family-friendly environment in which all faculty and staff can excel and achieve work/life balance irrespective of ethnicity, gender, faith, gender identity and expression as well as sexual orientation. A pre-employment background investigation is performed on candidates selected for employment. In addition, UAB Medicine maintains a drug-free and tobacco-free work environment. Physicians and other clinical faculty candidates, who will be employed by the University of Alabama Health Services Foundation (UAHSF) or other UAB Medicine entities, must successfully complete a pre-employment drug and nicotine screen to be hired. UAB also encourages applications from individuals with disabilities and veterans.



Texas State University-San Marcos is a member of the Texas State University System.

Assistant Professor Microbiology

The Department of Biology at Texas State University (www.bio.txstate.edu) invites applications for a tenure-track Assistant Professor in Microbiology. The successful candidate will be expected to teach both graduate and undergraduate courses in the Department of Biology and develop an externally funded research program that complements the existing strengths of our 34-member faculty. Required qualifications are a Ph.D. in Microbiology or related life sciences, postdoctoral experience, and a publication record in microbiology. Preferred qualifications are a record of extramural funding to support research, a record of interdisciplinary collaboration, and experience in research complementing the research strengths of the Department. The successful candidate is expected to teach one course per semester in virology or immunology. Salary and start-up package are negotiable.

Review of applications will begin **December 15, 2013** and continue until the position is filled. A letter of application with statements on research plans and teaching philosophy, CV, pdfs of five representative publications, and the names and contact information of five people willing to serve as references should be sent, as a single PDF, to microbiology@txstate.edu. Questions about this position should be addressed to **Dr. Robert McLean**, rm12@txstate.edu, Texas State University-San Marcos, 601 University Drive, San Marcos, TX 78666.

*Texas State University is an
Affirmative Action/Equal Opportunity Employer.*



Australian Government
Australian Research Council

FUTURE FELLOWSHIPS CALL FOR PROPOSALS

The Australian Research Council (ARC) is seeking proposals from outstanding national and international mid-career researchers, across all research disciplines.

The Future Fellowships scheme aims to attract and retain the world's best and brightest mid-career researchers, who can increase Australia's research capacity and build collaboration across institutions, industry and/or research disciplines.

From 1 July 2014, the ARC will award up to 150 four-year full-time fellowships at three salary levels: \$111,739, \$135,264 and \$158,787 (in Australian dollars) plus additional on-costs of 28 per cent. The ARC will also provide up to \$50,000 of non-salary funding per year which may be used for infrastructure, equipment, travel and relocation costs directly related to the research.

Future Fellowships must be applied through and held at an eligible Australian research organisation. The closing date for proposals is 20 November 2013.

Proposals from researchers working in areas of national priority and targeted priorities across all disciplines that will result in economic, environmental, social, health and/or cultural benefits for Australia are encouraged.

For further information, documentation and key dates regarding the Future Fellowships Scheme visit www.arc.gov.au or email ARC-FutureFellowships@arc.gov.au.

AG77017

RESEARCH

in the national interest - enabling the future



FACULTY POSITIONS IN DEVELOPMENTAL BIOLOGY AND/OR NEUROSCIENCE

The Department of Cell and Molecular Biology at Tulane University (<http://cell.tulane.edu/>) anticipates filling one or two tenure-track Assistant Professor position(s), beginning July 1, 2014. These positions are subject to final budgetary approval. Targeted are individuals whose research interests focus on Developmental Biology with an emphasis in mammalian organogenesis, or on neuroscience.

The Department is undergoing a rebuilding process and has targeted Neuroscience and Developmental Biology as areas for rapid growth. Applicants must have a Ph.D., at least 2 years of postdoctoral experience, a strong publication record and show strong potential for obtaining external funding. The successful applicant will be expected to establish a vigorous, independent research program and to participate in graduate and undergraduate teaching. Opportunities exist for research collaborations and participation in the Tulane Cancer Center, the Tulane Primate Center, the Center for Bioenvironmental Research, and the Tulane Neuroscience Program.

Applicants should send curriculum vitae, a brief statement of research interests and three letters of recommendation by **December 31, 2013** to:

Dr. YiPing Chen
Chair, Department of Cell and Molecular Biology
Tulane University
2000 Percival Stern Hall
New Orleans, LA 70118

Tulane University is an Equal Opportunity/Affirmative Action/ADA Employer and encourages minority and female applicants to apply.

Associate Director, Cancer Prevention & Control



The Medical College of Wisconsin (MCW) Cancer Center is seeking an Associate Director for Prevention & Control. The successful candidate will have an MD, PhD, or MD/PhD degree and a distinguished record of achievement in cancer epidemiology/risk assessment, behavioral and outcomes research, cancer prevention, or community engagement/cancer disparities. Rank and salary will be commensurate with the candidate's credentials and experience, including the potential for an endowed chair in cancer research.

The Associate Director is expected to provide strong leadership in the development of cancer prevention and control efforts in all Cancer Center programs with an emphasis on community engagement and cancer disparities across the Cancer Center's catchment area. The selected candidate will be a member of the senior leadership team of the Cancer Center and will report directly to the Center Director. The successful candidate will be expected to bring significant extramural funding, particularly NCI R or P grants, and demonstrate a successful track record of high quality publications and funded research.

Cancer is the top strategic priority at MCW. The Cancer Center is comprised of more than 200 cancer research scientists and physicians at MCW, Froedtert Hospital, Children's Hospital of Wisconsin, Clement Zablocki VA Medical Center, and the BloodCenter of Wisconsin, and has an ambitious plan for growth, including achieving NCI-designation. The MCW Cancer Center occupies 30,000 sq. ft. of space dedicated to cancer-related research and 50,000 sq. ft. at the Froedtert Clinical Cancer Center.

Qualified individuals are encouraged to send a letter of interest, CV, and contact information for three references via email to Michele Ward at cancer@mcw.edu, with the subject line "AD of Prevention & Control."

ATTN: AD of Prevention & Control Search Committee, c/o Michele Ward
Medical College of Wisconsin Cancer Center
8701 Watertown Plank Road, Milwaukee, WI 53226

COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK

Neuroscience Faculty Recruitment

The Department of Neuroscience at Columbia University is currently recruiting faculty in the neurosciences. We have a particular interest in research programs that link neural circuitry and behavior in genetically-tractable mammalian model systems. We will also consider candidates addressing issues in cognitive neuroscience at the level of systems and circuits.

Columbia University has an exceptionally strong and broad program in the neurosciences and aims to enhance interactions between basic and clinical research and to link the neurosciences with a wide range of other disciplines within the University. New faculty will be affiliated with the Department of Neuroscience, with the Doctoral Program in Neurobiology and Behavior, and with the newly established Zuckerman Mind Brain Behavior Institute. In addition, there are numerous opportunities for interaction with other scientific departments and programs at the Medical Center and Morningside Heights campuses.

The application deadline is November 30, 2013. Please submit applications online at <https://academicjobs.columbia.edu/applicants/Central?quickFind=58277> and include a cover letter, curriculum vitae, and a statement of research interests. In addition, please arrange for three references to submit letters of recommendation.

Columbia University is an affirmative action/
equal opportunity employer.

MAX-PLANCK-GESELLSCHAFT zur Förderung der Wissenschaften e.V.



MAX-PLANCK-GESELLSCHAFT

Max Planck Research Groups

The Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. is an independent, non-profit research organization, whose goal is to promote top quality research at its institutes. The over 80 research institutes of the Max-Planck-Gesellschaft conduct basic research in **Biology** and **Medicine**; **Chemistry**, **Physics** and **Technology**; the **Humanities**, **Social Sciences** and **Law**. In particular, the Max-Planck-Gesellschaft addresses new, innovative and interdisciplinary research areas.

To achieve this, we need you - outstanding postdocs in all fields of research pursued in our organisation. We invite you to work with us and apply for a position as Max Planck Research Group Leader. We also encourage candidates with an interdisciplinary background and those who are working in the field of Astrobiology and Origins of Life.

Excellent research is irrespective of gender. However, the Max-Planck-Gesellschaft still lacks female scientists and their outstanding talents. We hereby explicitly welcome applications from highly skilled women in all disciplines.

The successful candidates will be offered a **Max Planck Research Group** for a period of five years **at a Max Planck Institute of their own choice**. This includes a V2 position equivalent to assistant or associate professor level and additional resources for research positions, budget, and investments. The cumulative amount of funding is competitive with top class start-up packages of international career development programs. Start of funding is in 2015, with a lead time of up to three months.

Your applications should include a CV, a list of publications, copies of three publications, a one-page summary of scientific achievements, two letters of recommendation, and a two-page research plan.

For further information and detailed application instructions please see

<http://www.mprg.mpg.de>

The Max-Planck-Gesellschaft is committed to employing individuals with disabilities and explicitly encourages them to apply.

The deadline for application is **November 24, 2013**.



Worldwide Search for Talent

City University of Hong Kong is a dynamic, fast-growing university that is pursuing excellence in research and professional education. As a publicly-funded institution, the University is committed to nurturing and developing students' talent and creating applicable knowledge to support social and economic advancement. Currently, the University has six Colleges/Schools. Within the next two years, the University aims to recruit **100 more scholars** from all over the world in various disciplines, including **science, engineering, business, creative media, energy, environment, humanities, law, social sciences**, and other strategic growth areas.

Applications and nominations are invited for:

Dean of College of Science and Engineering

[Ref. A/125/29]

The Position

Reporting to the Provost, the Dean will provide visionary, strategic leadership for the College, in alignment with the vision, mission and Strategic Plan of the University. In collaboration with the College's faculty and other academic units, the Dean will advance the College's academic excellence in research and professional education, and further its international standing.

The Person

The individual shall possess a doctorate degree and have strong academic and professional qualifications, a distinguished record of teaching, research and scholarship, and substantial relevant experience in academic leadership roles; the vision and capability to build on and expand the College's strengths; outstanding management effectiveness; commitment to interdisciplinary collaboration and teamwork; and strong communication and networking skills to build and nurture internal and external contacts to the benefit of the College and the University.

Salary and Conditions of Service

The appointee will be offered appointment to an academic rank commensurate with qualifications and experience. The deanship appointment will be on a concurrent basis for an initial period of three years and renewable for another three years subject to mutual agreement. Remuneration package will be attractive and driven by market competitiveness and individual performance. Excellent fringe benefits include gratuity, leave, medical and dental schemes, and relocation assistance (where applicable).

Information and Application

Further information on the post and the University is available at <http://www.cityu.edu.hk>, or from the Human Resources Office, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong [Email: deancse@cityu.edu.hk/Fax: (852) 2788 1154 or (852) 3442 0311].

Please send the nomination or application with a current curriculum vitae and a statement on suitability and vision to serve as Dean to Human Resources Office, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong, or e-mail to "deancse@cityu.edu.hk". Please quote the reference of the post in the application and on the envelope. **Applications and nominations received before 15 November 2013 will receive full consideration.** The University's privacy policy is available on the homepage. The University reserves the right not to fill the position. Personal data provided by applicants will be used strictly in accordance with the University's personal data policy, a copy of which will be provided upon request.

The University also offers a number of visiting positions through its "CityU International Transition Team" scheme for current graduate students, postdoctoral scholars, and for early-stage and established scholars, as described at http://www.cityu.edu.hk/provost/cityu_international_transition.htm.

City University of Hong Kong is an equal opportunity employer and we are committed to the principle of diversity. We encourage applications from all qualified candidates, especially those who will enhance the diversity of our staff.

City University of Hong Kong was ranked 5th among the world's top 50 universities under the age of 50 in the *Quacquarelli Symonds* 2013 survey.
<http://www.cityu.edu.hk>



Department of Biological Sciences
Assistant Professor
Cellular Biophysics
and Cellular Biology

The Division of Natural Sciences and Mathematics at the University of Denver is expanding our Molecular Life Sciences Initiative with 10 tenure-track hires in recent years and 5 more in the next two years. The Department of Biological Sciences invites applicants for two tenure track faculty positions at the Assistant Professor level to begin September 1, 2014. We are seeking candidates with research interests in cellular biophysics and related areas of cell biology that include but are not limited to the study of intracellular signaling and trafficking, protein-protein interactions, and cell physiology. Individuals applying advanced fluorescence imaging or electrophysiology are encouraged to apply. The successful candidate will have a Ph.D. and post-doctoral experience in an appropriate field, will develop an extramurally funded research program, will supervise Ph.D. and M.S. students and undergraduate research projects and will teach undergraduate and graduate courses in area of expertise. All candidates must submit their application through www.du.edu/hr/employment/jobs.html. Information on Departmental programs can be found at <http://www.du.edu/nsm/departments/biologicalsciences/>. Successful applicants will also be eligible to participate in the interdepartmental Molecular and Cellular Biophysics Ph.D. program <http://www.du.edu/nsm/departments/molecularandcellular/index.html>.

The online application should include: a curriculum vitae, and statements of research interests and teaching philosophy and two recent publications. In addition, at least three recommenders should email letters of reference to: **Faculty Search Committee, University of Denver, Department of Biological Sciences at biology.rec@du.edu**. The review of applications will begin **November 15, 2013** and continue until the position is filled. Contact **Professor David Patterson at dpatter2@du.edu** if you have questions regarding the search.

The University of Denver is committed to enhancing the diversity of its faculty and staff and encourages applications from women, minorities, members of the LGBT community, people with disabilities and veterans. The University is an Equal Opportunity/Affirmative Action Employer.



FACULTY POSITION in Biomolecular Catalysis
Department of Biochemistry

As part of an on-going programmatic initiative, the Department of Biochemistry at the University of California, Riverside invites applications for a new faculty position at the Assistant Professor level in the general area of biomolecular catalysis, specifically including (but not limited to) areas of Enzyme Dynamics, Metalloenzymology, RNA Catalysis, and the Enzymology of DNA/Histone Modification. Individuals utilizing bioinformatics approaches to identifying promising new targets for study are especially encouraged to apply. The successful candidate will be expected to develop a vigorous, independent, and innovative research program that is able to attract extramural funding, and also to contribute to the Department's undergraduate and graduate teaching missions. The position is part of a continuing interdisciplinary effort at UCR to expand biomolecular catalysis and molecular biophysics on campus, particularly in areas of biomedical relevance. A competitive start-up package will be provided, with salary commensurate with education and experience. The position is available July 1, 2014. A Ph.D., M.D., or equivalent degree is required.

Interested individuals should apply at <https://aprecruit.ucr.edu/apply/JPF00006>. A *curriculum vitae*, a brief statement of research interests, and three letters of reference are required.

The University of California, Riverside is recognized as one of the nation's most ethnically and culturally diverse campuses, and applications from highly qualified women and underrepresented minorities are particularly encouraged. Review of applications will begin **November 15, 2013** and will continue until the position is filled. For additional information about the Department, visit <http://biochemistry.ucr.edu>, and for the campus, visit <http://www.ucr.edu>.

The University of California is an Affirmative Action/Equal Opportunity Employer.



UNIVERSITY OF CALIFORNIA
 SANTA BARBARA

Faculty Position in Quantitative Systems Biology
Division of Mathematical, Life and Physical Sciences

The University of California Santa Barbara invites applications for a tenure-track faculty position at the Assistant Professor Level. This position is a part of a multi-year, multi-departmental initiative to establish an interdisciplinary program in the area of Quantitative Systems Biology, emphasizing quantitative experimentation and theoretical analysis in the study of living systems. Subjects of interest include, but are not limited to: quantitative studies of development, microbial biology and dynamics of evolution as well as population genetics and genomics. While faculty may be associated with more than one department, the primary appointment would be in the academic unit most closely corresponding to the candidate's research focus and teaching qualifications. Preference will be given to candidates with broad scientific interests, a record of research excellence and creativity and a strong commitment to undergraduate and graduate teaching. Interdisciplinary educational background combining biology with physical and mathematical sciences and/or a strong record of active interdisciplinary collaboration will also be valued.

The university is especially interested in candidates who can contribute to the diversity and excellence of the academic community through research, teaching and service. Candidates must possess a Ph.D. by September 2014. Appointment begins July 1, 2014.

Applications must include a statement of research interests, a curriculum vitae, and a list of publications. Applicants should arrange for at least three letters of recommendation. Materials should be submitted electronically via <https://recruit.ap.ucsb.edu/apply>. Applications are due **January 15, 2014** for primary consideration, but the position will remain open until filled. Questions can be emailed to qbio-search@lifesci.ucsb.edu.

UCSB is an Equal Opportunity/Affirmative Action Employer.

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 that answers questions.



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HUMAN FRONTIER SCIENCE PROGRAM

www.hfsp.org

POSITION OF SECRETARY GENERAL

The Board of Trustees of the Human Frontier Science Program (HFSP), representing the member countries*, invites applications and nominations for the position of Secretary General of HFSP. The SG is responsible for the implementation of the Program and is the public voice for HFSP in the world of global science funding. HFSP supports research into the complex mechanisms of living organisms, promoting international collaboration in the spirit of science without borders. It offers funding programs that enable innovative, cutting-edge and high-risk research at the frontiers of the life sciences and actively fosters the financial and intellectual independence of early-career researchers. The SG therefore should be:

- An internationally recognized and respected scientist, preferably with experience of senior-level scientific management, who is convinced of the distinctive value of international collaboration and is therefore able to make a compelling case for the support and promotion of frontier research globally, strengthening this prestigious Program and leading it into the next period of its development;
- An innovative researcher, qualified in a field relevant to the scientific priorities of the Program, who understands the importance of novel approaches for the development of life-science research.

The SG will be based in Strasbourg, France for the duration of the appointment, which begins July 2015 for a renewable term of 3 years. Candidates must be citizens of an HFSP member country.

Applications and nominations, accompanied by a CV, should be sent to SecGen@hfsp.org by **30 November 2013**. Inquires, nominations, and applications may also be addressed to HFSP's search consultant, Craig Smith, PhD, of Opus Partners: craig.smith@opuspartners.net.

*Member countries are Australia, Canada, France, Germany, India, Italy, Japan, Korea (Republic of), New Zealand, Norway, Switzerland, the UK, USA and EU.



DANA-FARBER
CANCER INSTITUTE

Assistant/Associate Professor of Medicine Cancer Geneticist/Computational Biologist

The Dana-Farber Cancer Institute, Brigham and Women's Hospital and the Department of Medicine, Harvard Medical School, invite applications from cancer geneticists or computational biologists with a focus in cancer genetics for full-time appointment at the Assistant/Associate Professor level. This individual will develop an independent research program focused on the contribution of the germline to the basis of inherited cancer susceptibility and/or pharmacogenomics. Population scientists, computational biologists and laboratory-based investigators are encouraged to apply. The successful candidate should be committed to the development of an innovative scientific research program and to collaboration with the faculty at the Dana-Farber and Harvard Medical School interested in the role of the germline in cancer susceptibility and/or pharmacogenomics. The candidate will be based in the Divisions of Population Sciences and Molecular and Cellular Oncology in the Department of Medical Oncology at the Dana-Farber Cancer Institute, and will be a member of the Department of Medicine at the Brigham and Women's Hospital and Harvard Medical School.

This position includes a highly competitive compensation and start-up package. Applicants must have an MD and/or PhD and a proven track record of outstanding research.

Interested candidates should submit a curriculum vitae, brief research overview and three letters of reference to Dr. Judy Garber, Director, Center for Cancer Genetics and Prevention, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215. Submissions may be sent via email to: DFCI_genetics_search@dfci.harvard.edu. Please direct telephone inquiries to Andreina Viera-Reyes at 617-632-5770.

The search is being conducted jointly with Dr. William Hahn, Deputy Chief Scientific Officer, and Chief, Division of Molecular and Cellular Oncology, Dana-Farber Cancer Institute.



HARVARD
MEDICAL SCHOOL



BRIGHAM AND
WOMEN'S HOSPITAL

Dana-Farber Cancer Institute/Brigham and Women's Hospital/Harvard Medical School are Equal Opportunity/Affirmative Action Employers actively committed to increasing the diversity of our faculty: people with disabilities, veterans, women and members of underrepresented minority groups are therefore strongly encouraged to apply.

Worcester Polytechnic Institute

Endowed Chair in Biochemistry

Worcester Polytechnic Institute (WPI) invites applications and nominations of candidates for the Richard T. Whitcomb Professorship in Biochemistry. The holder of the Whitcomb chair will be a dynamic scholar and teacher, with a strong track record of creativity and an internationally highly visible research program, studying biochemical systems at the molecular level. The successful candidate will have a strong record of continued funding, high impact publications, and a solid presence in the scientific community. This Professorship offers a generous start-up package, continued financial support for research and substantial laboratory space in the recently built, state-of-the-art Life Sciences research facility.

The appointment will be made in the Department of Chemistry and Biochemistry (CBC), within Arts and Sciences at WPI. The Department offers undergraduate and graduate (MS and PhD) degrees in Chemistry and Biochemistry. CBC is integral to WPI's ongoing major life science research initiative and five new faculty at the assistant, associate and full professor level were recently hired. The ideal candidate for the Whitcomb Professorship will have a strong interest in collaborative research and is expected to provide leadership in new or established research areas at the interface of Chemistry and Biology.

Further information about WPI and the Department of Chemistry and Biochemistry can be found at wpi.edu/+chemistry.

Applicants are asked to apply electronically by visiting Careers at WPI; <http://apptrkr.com/398704>. Review of applications will commence immediately and continue until the position is filled. Inquiries may be addressed to Dr. José Argüello (arguello@wpi.edu) Whitcomb Search Committee, Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, 100 Institute Road, Worcester, MA 01609.



WPI

To enrich education through diversity, WPI is an Affirmative Action, Equal Opportunity Employer.

UC DAVIS

**Assistant Professor in Plant Sciences
Computational and
Statistical Plant Biology
Department of Plant Sciences
Assistant Professor in Computational
and Statistical Plant Biology**

The Department of Plant Sciences in the College of Agricultural and Environmental Sciences at the University of California Davis invites applications for a 9-month tenure-track faculty appointment at the Assistant Professor level in Computational and Statistical Plant Biology. We seek candidates with a strong background in statistics and/or computational biology working in areas such as bioinformatics, quantitative genetics and genomic selection, systems biology, or ecological, evolutionary, functional, or comparative genomics. In particular we are interested in candidates whose research seeks to develop and apply quantitative methods to interrogate large data sets to improve our understanding of the connection between genotype, phenotype, and the environment. The successful candidate will have a PhD in a related discipline, preferably with postdoctoral experience. She or he will be expected to teach upper division undergraduate and graduate courses in the areas of bioinformatics, statistical analysis, and experimental design; to establish an extramurally funded research program; to mentor and train students and postdoctoral scholars; and to interact with a diverse faculty including plant geneticists, breeders, physiologists, and ecologists. This position will include an appointment in the Agricultural Experiment Station, which includes the responsibility to conduct research and outreach relevant to the mission of the California Agricultural Experiment Station.

QUALIFICATIONS: Ph.D. or equivalent level of experience in Bioinformatics, Computational Biology, Statistics, Quantitative or Populations Genetics or related fields.

SALARY: Commensurate with qualifications and experience.

TO APPLY: Candidates should begin the application process by registering online at <http://recruitments.plantsciences.ucdavis.edu/>. Please include statements of research and teaching interests, curriculum vitae, publication list, copies of 3 of your most important research publications, copies of undergraduate and graduate transcripts (if within 5 years of either degree), and the names, e-mail addresses, and telephone numbers of at least 4 professional references. For technical or administrative questions regarding the application process, please email bkniijar@ucdavis.edu. Review of the applications will begin **December 15, 2013**. The position will remain open until filled. Please direct inquiries to:

**Dr. Jeffrey Ross-Ibarra, Chair,
Search Committee
Department of Plant Sciences
University of California
One Shields Avenue
Davis, CA 95616-8515
Telephone: (530) 752-1152
E-mail: rossibarra@ucdavis.edu**

The University of California, Davis, and the Department of Plant Sciences are interested in candidates who are committed to the highest standards of scholarship and professional activities, and to the development of a campus climate that supports equality and diversity. The University of California, Davis is an affirmative action/equal opportunity employer with a strong institutional commitment to the achievement of diversity among its faculty and staff. UC Davis is an NSF ADVANCE institution committed to equality and inclusion.

POSITIONS OPEN



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UNIVERSITY®**

Temple University School of Medicine (TUSM)/Center for Metabolic Research Center (CMDR) seeks to recruit basic science researcher for tenure-tracked department faculty positions at the **ASSISTANT** or **ASSOCIATE PROFESSOR** levels, depending on qualifications. The applicant will have M.D., Ph.D., or M.D.-Ph.D. degrees, have completed rigorous training and have the ability to develop an independent extramurally funded laboratory. The ideal candidate will have expertise and have applied modern approaches in studying biochemistry and molecular biology basis of metabolic disease, including hyperlipidemia, hyperhomocysteinemia, hyperglycemia, obesity, diabetes, uremia, and metabolic syndrome. Candidates are asked to send their applications (including curriculum vitae, a concise research summary, and names and contact information for three references) to: **Dr. Hong Wang, M.D., Ph.D., EMBA, Director, CMDR, Temple University School of Medicine, 3500 North Broad Street, Philadelphia, PA 19140 (e-mail: hong.wang@temple.edu)**

Temple University is an Affirmative Action/Equal Opportunity Employer and strongly encourages applications from women and minorities.

FACULTY POSITION

The Department of Molecular and Cell Biology at the Boston University Henry M. Goldman School of Dental Medicine has an opening at the **ASSISTANT, ASSOCIATE, or FULL PROFESSOR** level. Individuals with an outstanding publication record and an ongoing NIH R01-funded research program are encouraged to apply. We seek qualified candidates with research interests in cell biology, developmental biology, molecular genetics, biochemistry, immunology, or microbiology. Expertise in craniofacial and/or oral biology is preferred but not required. Excellent laboratory facilities and start-up funds are available as well as joint appointments with appropriate departments at the School of Medicine and participation in the Bioinformatics Program at the School of Engineering. Qualified applicants should submit a curriculum vitae, a brief summary of research accomplishments, a description of plans for future work, and names of three to five references, by December 15, 2013. Electronically send all materials to **Dr. P. C. Trackman, Search Committee Chair at e-mail: mcbdept@bu.edu**.

Please visit website: <http://dentalschool.bu.edu/research/molecular/index.html>.

Boston University is an Affirmative Action/Equal Opportunity Employer.

ASSISTANT PROFESSOR Computational Biologist/Tenure-track Department of Biological Sciences Louisiana State University

This is a tenured-track faculty position and the successful candidate will be expected to establish a vigorous, extramurally funded research program in Computational Genomics at Louisiana State University (LSU), and contribute to undergraduate and graduate teaching. The candidate will have an opportunity to interact with and participate in the Center for Computation & Technology (CCT). CCT offers an innovative and interdisciplinary research environment for advancing computational sciences. Required Qualifications: Ph.D. and a successful track record of independent research. An offer of employment is contingent on a satisfactory pre-employment background check. Application deadline is December 13, 2013 or until a candidate is selected. Apply online and view a more detailed ad at website: <http://www.lsusystemcareers.lsu.edu>. Position: 031162.

Louisiana State University is an Equal Opportunity/Equal Access Employer

Quick link at ad website: <https://lsusystemcareers.lsu.edu/applicants/Central?quickFind=56690>.

POSITIONS OPEN



**Perelman
School of Medicine
UNIVERSITY OF PENNSYLVANIA**

ASSISTANT PROFESSOR OF PHYSIOLOGY

The Department of Physiology at the Perelman School of Medicine at the University of Pennsylvania seeks candidates for an Assistant Professor position in the tenure track. Responsibilities include establishing and conducting an independent research program, and supervising, mentoring, and teaching students. Applicants must have an M.D., Ph.D., or equivalent degree and have demonstrated excellent qualifications in research.

The successful applicant will have experience in any aspect of physiology, ranging from integrative to cell biological to molecular biophysical. Emphasis will be placed on novelty and impact of the research, with special consideration given to candidates employing cutting-edge approaches. Please submit a cover letter, curriculum vitae, the names and contact information of three references, and a two- to four-page statement of your research interests.

First consideration will be given to applications received by November 30, 2013, but applications will continue to be accepted after this date.

We seek candidates who embrace and reflect diversity in the broadest sense. The University of Pennsylvania is an Equal Opportunity/Affirmative Action Employer.

Apply for this position online only at website: http://www.med.upenn.edu/apps/faculty_ad/index.php/g311/d3426.

ASSISTANT PROFESSOR-ALL AREAS Princeton University Department of Chemistry

The Department of Chemistry at Princeton University invites applications for a tenure-track assistant professor position in all areas of chemistry. We seek faculty members who will create a climate that embraces excellence and diversity, with a strong commitment to teaching and mentoring that will enhance the work of the department and attract and retain students of all races, nationalities, and genders. We strongly encourage applications from members of all underrepresented groups. Candidates are expected to have completed the Ph.D. in chemistry or a related field at the time of appointment. Applicants should submit a description of research interests, curriculum vitae, a list of publications, and contact information for three references online at website: <http://jobs.princeton.edu/applicants/Central?quickFind=64207>. The search committee will begin review of applications on October 17, 2013 and will continue until the position is filled.

Princeton University is an Equal Opportunity Employer and complies with applicable EEO and Affirmative Action regulations.

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